
THE UNIVERSITY *of* EDINBURGH

# The role of plant growth promoting bacteria and a leguminous plant in metal sequestration from metal contaminated environments by *Brassica juncea*


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## Declaration

I declare that this thesis was composed by myself and has not been submitted for any other degree. The work described is my own, unless otherwise indicated.

Gbotemi A. Adediran

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## Dedication

To my wife, Oluwafunmilola Mercy Adediran
and my late Mum, Alake Maria Adediran

*"Within one linear centimetre of your lower colon there lives and works more bacteria (about 100 billion) than all humans who have ever been born. Yet many people continue to assert that it is we who are in charge of the world."*

- **Neil Degrasse Tyson**

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## Abstract

The worldwide occurrence of sites contaminated with toxic metals and the associated high costs of remediating them using chemical and mechanical methods have led to calls to develop inexpensive and sustainable approaches based on the use of plants that naturally accumulate large amounts of metals in their tissues. The ability of plants to remediate metals has been rigorously studied and some species have been identified as excellent phytoremediators. However, the growth of phytoremediators is often retarded under high soil metal concentrations, rendering them ineffective. Meanwhile, some plants do not have remediating abilities but are capable of growing in contaminated environments with little or no sign of stress. Despite the volume of research dedicated to the screening and evaluation of phytoremediators, major questions remain about why some plants survive but do not remediate while the growth of phytoremediators is mostly hindered.

The growth and metal-remediating efficiency of plants exposed to toxic concentrations of metals can be enhanced by inoculating phytoremediating plants with certain bacteria but the mechanisms behind this process remain unclear. Furthermore, the use of leguminous plants to improve the growth of a target plant under a mixed planting system has long been recognised as an effective yield-enhancing cropping system. However, the possibility of a non-remediating but tolerant leguminous plant conferring metal tolerance to a phytoremediator has not been explored.

This thesis reports results from repeated glasshouse and lab-based growth experiments on the phytoremediating plant *Brassica juncea* exposed to

400 – 600 mg Zn $kg^{-1}$. The aim was to investigate the abilities of two plant growth promoting bacteria (PGPB) species *Pseudomonas brassicacearum* and *Rhizobium leguminosarum,* and a leguminous plant *Vicia sativa* to promote *B. juncea* growth and enhance remediation of Zn-contaminated soil. *B. juncea* plant roots were analysed using synchrotron based micro-focus X-ray Fluorescence (μXRF) imaging and X-ray Absorption Near Edge Structure (μXANES) analysis to probe Zn speciation.

*P. brassicacearum* exhibited the poorest plant growth promoting ability, while *R. leguminosarum* alone and in combination with *P. brassicacearum* significantly enhanced *B. juncea* growth and Zn bioaccumulation. X-ray Absorption Spectroscopy (XAS) analysis showed that reduced plant growth was due to root accumulation of Zn as Zn sulphate, Zn oxalate and Zn polygalacturonic acids. The better growth and increased metal accumulation observed in plants inoculated with *R. leguminosarum* and its combination with *P. brassicacearum* was attributed to root storage of Zn in the chelated forms of Zn phytate and Zn cysteine. A subcellular analysis of plant root also showed that the PGPB enhanced tolerance to Zn contamination by enhancing epidermal Zn compartmentalisation depending on the nature of root colonization, and induced changes in Zn speciation to less toxic Zn species in the epidermis and endodermis of plant root. The thesis therefore identifies enhanced Zn compartmentalization at the root epidermis and bacterial mediated changes in Zn toxicity through changes in Zn speciation as key complimentary mechanisms of plant growth promotion and enhanced Zn accumulation in plants by PGPB.

Further experiments investigating alternative phytoremediation strategies showed that the use of the leguminous plant *V. sativa* in a mixed planting system with *B. juncea* plants completely out performed the effects of bacteria in promoting the growth and remediation potential of *B. juncea* under Zn contamination. By combining PGPB with mixed planting, *B. juncea* recovered full growth while also achieving maximum phytoremediation efficiency. The novel legume assisted-microbial phytoremediation method that is reported in this thesis is the first to demonstrate complete plant growth recovery in plants exposed to 400 – 450 mg $kg^{-1}$ soil Zn contamination for 5 weeks. Survival of *V. sativa* was attributed to its root storage of Zn in the chelated forms of Zn histidine and cysteine whereas in the roots of stunted *B. juncea* plants the majority of Zn was present as Zn oxalate and toxic Zn sulphate. Although the use of natural and synthetic chelates has been reported to enhance phytoremediation, this thesis recommends a legume-assisted-microbial-phytoremediation system as a more sustainable method for Zn bioremediation.

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Chapter 1

# 1. Introduction to thesis

## 1.1. Research rationale

Metals and metalloids like cadmium, zinc, chromium, lead and arsenic are poisonous elements that are widely associated with environmental contamination (Peralta-Videa et al., 2009, Gil et al., 2011, Cao et al., 2014). They are mainly released into the environment through anthropogenic activities like mining and smelting of metalliferous ores, electroplating, energy and fossil fuel production, fertiliser and agrochemicals application (Alkorta et al., 2004). They are non-biodegradable and thus readily accumulate to toxic levels in the environment (Kumar Sharma et al., 2007). The extent of metal contaminated sites worldwide (Nriagu and Pacyna, 1988, Li et al., 2001, Lado et al., 2008) and the potential mobilisation of metal contaminants from soil to surface and ground water and bioaccumulation in edible crops and feeds (Alam et al., 2002, Lui et al., 2006), have necessitated the development of chemical and mechanical remediation methods (Mulligan et al., 2001a, Dermont et al., 2008).

These methods mainly approach the remediation of metal contaminated sites through excavation and landfilling of contaminated soil, thermal treatment, acid leaching and electro-reclamation (Jing et al., 2007, Zhang et al., 2013a). Although these remediation methods have been effective, they have been found environmentally destructive, expensive and unsustainable (Mulligan et al., 2001a, Wu et al., 2010).

By contrast, plants are natural miners of nutrients and other elements from the environment (Sheoran et al., 2009). The unique ability of some plants to significantly

remediate metal contaminated environments has been rigorously researched and some species have been identified as excellent metal phytoremediators (McGrath and Zhao, 2003, Vara Prasad and de Oliveira Freitas, 2003).

Some plants do not have remediating abilities but are capable of growing on contaminated environment with little or no sign of stress from metal toxicity (Kováčik et al., 2006, Broadley et al., 2007); in contrast, the growth of metal remediating plants is often retarded under high soil metal contamination (Ebbs and Kochian, 1997, Lombi et al., 2001). Often these phytoremediators die before having any significant remediation effect. Despite the volumes of research dedicated to the screening and evaluation of phytoremediators, the reason why some plants survive but do not remediate and why the growth of phytoremediators is mostly hindered, remain poorly understood.

Moreover, the growth and metal-remediating efficiency of phytoremediators exposed to high metal concentrations has been found to be enhanced by inoculating the plants with certain bacteria (Belimov et al., 2005, Ma et al., 2009, Ma et al., 2011a). However, the mechanisms behind enhanced plant growth in the face of high metal bioaccumulation remain debatable. While the role(s) of bacteria in enhancing plant growth and metal sequestration is still being researched, there is no consensus on the bacteria types or species that are more suitable for promoting plant growth under soil metal contamination. For example, inoculating a phytoremediator with a native endophytic bacteria isolated from the same plant has been suggested as a good strategy for efficient microbial-phytoremediation (Ma et al., 2011b). However, the use of rhizospheric nutrient fixing and releasing bacteria, which are not necessarily

sourced from the same phytoremediator, has also been proposed as ideal (Zhuang et al., 2007).

Furthermore, the use of leguminous plants to improve the growth of a target plant under a mixed planting system has been evaluated and accepted as a crop production system in nutrient deficient soils (Ghosh et al., 2007, Malézieux et al., 2009). However, the possibility of a non-remediating but metal tolerant plant conferring its survival ability to a phytoremediator under metal toxicity has not been explored.

This thesis evaluates novel use of plants and bacteria for remediation of metal contaminated soil. A range of techniques, from pot experiments to synchrotron based micro-X-ray Absorption Spectroscopy (μ-XAS) were utilised to investigate some of the mechanisms underpinning metal tolerance and phytoextraction and bioaccumulation by plants.

### 1.2. Research scope

The goal of this PhD study is to develop novel and sustainable bioremediation strategies that remediates contaminated soil using a combination of plants and plant growth promoting bacteria (PGPB). Most importantly, it aims to unravel the constraining mechanism(s) behind increase in plant growth in the face of increased toxic metal accumulation in plants inoculated with bacteria or mixed planted with a leguminous plant. Different scientific opinions exist with respect to the role of bacteria in microbial assisted phytoremediation. Bacterial improvement in plant nutrient provision factors (addition or release of essential nutrients) and secretion of plant growth promoting factors (phytohormones and plant enzymes) by bacteria have been identified as likely roles of bacteria, but possible bacteria induced attenuation of

metal toxicity in plants has not been given due research consideration. A key focus of this study therefore, was to test whether plant growth promotion under metal contamination is linked to changes in metal speciation in plant tissue as a result of bacterial inoculation rather on the nutritional roles of the bacterial strain.

The majority of previous studies utilised destructive analytical techniques, such as chemical extraction of metals, hormones, enzymes and nutrients from bacteria and plants. Most studies have also broken down the metal-bacteria-plant system and studied each entity separately before drawing inferences which may not represent the dynamic biochemical reactions integrated within the whole metal-bacteria-plant system. This PhD however utilised synchrotron based micro X-ray Absorption Spectroscopy, coupled with the use of Confocal Laser Scanning Microscopy to study the nature of bacterial colonisation, metal localisation and metal speciation in fresh plant biomass exposed to metal contaminants. It also explores legume assisted phytoremediation as a more effective and sustainable method for remediating metal contaminated soil.

### 1.3. Research hypotheses

By choosing zinc as the metal contaminant, *Pseudomonas brassicacearum* and *Rhizobium leguminosarum* as the PGPB, *Vicia sativa* as the leguminous plant, and the well-known *Brassica juncea* as the phytoremediating plant; the following hypotheses were tested in this research.

(i) *P. brassicacearum* will promote the growth of *B. juncea* under Zn contamination compared to un-inoculated controls, based on it being a native bacteria strain isolated from a *Brassica* plant, which should facilitate root colonisation (Achouak et al., 2000).

(ii) *R. leguminosarum* will enhance *B. juncea* plant growth and survival under Zn contamination compared to un-inoculated controls, based on it being a well-known rhizobacterium of many plant species (both leguminous and non-leguminous) and for its reported ability to promote the growth of other *Brassica* plant species – *Brassica campestris and Brassica napus* (Noel et al., 1996, Schloter et al., 1997).

(iii) A combination of the two bacterial strains will confer multiple plant growth promoting effects under Zn contamination through a combination of their plant growth promoting abilities. i.e. more growth promotion when combination of both bacterial strains is used compared to single bacterial inoculation.

(iv) The bacterial strains will increase Zn bioaccumulation and soil Zn remediation by promoting *B. juncea* plant growth and inducing changes in Zn speciation in the *B. juncea* plant.

(v) There will be differences in Zn speciation between the epidermis and endodermis of *B. juncea* root due to the differences in the nature and properties of the root cell wall and the vacuole (Clemens, 2006, Lang and Wernitznig, 2011).

(vi) The two bacterial strains will co-localise with Zn at the root epidermis and enhance epidermal Zn sequestration, since higher root exudation and

bacterial population are mostly at the root epidermis compared to the root endodermis (el Zahar Haichar et al., 2008, Compant et al., 2010).

(vii) There will be differences in the nature of bacteria-metal co-localisation in *B. juncea* root between *P. brassicacearum* and *R. leguminosarum* since the former is a native endophytic bacterial strain of Brassica roots and the latter is a rhizospheric bacterial strain isolated from the root of a clover plant.

(viii) Better growth under Zn contamination in *B. juncea* inoculated with the PGPB will be due to sub-cellular storage of Zn as less toxic Zn chelates in plant roots.

(ix) The difference in Zn tolerance between *V. sativa* (a plant established in the course of the research to be of low Zn remediating ability but hyper-tolerant to Zn) and *B. juncea* (phytoremediating plant but less tolerant to Zn compared to *V. sativa*) will be due to differences in the nature of Zn species synthesised in the roots of the plants.

(x) Co-planting *B. juncea* with leguminous plant *V. sativa* will yield better *B. juncea* plant growth and soil Zn phytoremediation compared to the use of bacteria due to possible ability of the legume to confer its inherent resistance to the *B. juncea* under a mixed planting system.

### 1.4. Research objectives and methods

The specific objectives alongside the methods used to address them are presented in Table 1.1. Note that detailed description of methods is presented in Chapter 3 and

Chapter (s) where results of research objectives are presented is mentioned in Table 1.1.

**Table 1.1: Research objectives and methodology**

| Research objectives | Research methods |
|---|---|
| To determine the resistance/tolerance of the selected bacteria strains to zinc contamination. | Assessment of viable bacteria colony forming units after exposure to different concentrations of zinc contamination. (Chapter 4) |
| To characterise seed germination and seedling growth under metal contamination. | Seed germination assay and quantitative measurement of seedlings growth parameters (Chapter 7) |
| To determine bacteria survival and colonisation of roots in plant exposed to toxic metals under sterile condition and in glasshouse experiment | Scanning Electron Microscope imaging and the use of fluorophores with Confocal Laser Scanning Microscopy techniques (CLSM) (Chapter 4 and Chapter 7) |
| To determine the plant growth promoting ability of bacteria in plants exposed to toxic metals under sterile conditions and in glasshouse experiment | Quantitative measurements of growth parameters – plant height, plant root and stem length, and below and above ground dry plant biomass (Chapter 4, 5 and 7) |
| To investigate enhanced plant growth and phytoremediation in a phytoremediating plant exposed to zinc contamination, using bacteria and a leguminous plant as growth promoting agents. | Glasshouse factorial experiment: involving soil spiking with metal, seed inoculation with bacteria, phytoremediator-legume mixed planting, plant height and biomass assessment, pre and post experimental soil, plant analysis. (Chapter 5 and 6) |
| To investigate mechanism behind high remediation but poor metal tolerance ability of a remediating plant, in contrast to a hyper-tolerant but poor remediating leguminous plant | X-ray Absorption spectrophotometry (XAS) (Beamline I18, Diamond Light Source UK) (Chapter 5 and 6) |
| To investigate bacteria-Zn co-localisation and sub-cellular Zn speciation in plant root | Combine XAS and CLSM analysis (Chapter 7) |

### 1.5. Thesis structure

The structure of the thesis is as follows. Chapter 2 introduces the significance of metals as important ecosystems contaminants, providing an overview of literature on the behavior of metals in soil, water and plant systems, highlighting permissible limits and standards used in metal toxicity assessment and regulation, and discussing sustainable metal remediation methods. Chapter 3 presents the experimental designs and methods used to study bacteria and plant tolerance under metal toxicity, plant growth promotion by bacteria and legumes and the use of confocal microscopy and XAS spectroscopy to probe metal-bacteria co-localisation and speciation in plants.

Chapters 4-7 are data driven and have been prepared for submission for publications. Chapter 4 tested hypothesis (i), (ii) and (iii), reports results of plant growth promotion by bacteria from pot experiments conducted in a glasshouse. Chapter 5, tested hypothesis (iv), reports the biochemical mechanisms behind bacteria induced increase in plant growth and Zn bioaccumulation from pot experiments conducted in a glasshouse. Chapter 6 tested hypothesis (ix) and (x), reports legume-assisted-microbial-phytoremediation as a more efficient and sustainable remediation technique for soil contaminated with Zn. It also report results from glasshouse pot experiment. Chapter 7 tested hypotheses (v), (vi), (vii) and (vii), describes the phenomenon of bacteria-metal co-localisation and Zn speciation in laboratory based growth experiments established under aseptic experimental conditions. Finally in Chapter 8, the research findings are summarised and recommendations made for future research to further develop bioremediation as an economically viable and effective remediation technology for metal contaminated soil.

Chapter 2

# 2. Literature review

## 2.1. Introduction

The presence of highly toxic and persistent metal contaminants that have accumulated in the environment over a period of time is of significant global environmental concern. The ability of these metal contaminants to maintain their potency across the three environmental media of air, land and water and eventually poison the food chain, poses severe threats to health as well as the wellbeing of ecosystems. Heavy metals ingested through food or water, or inhaled, have been widely reported as one of the major causes of cancer, cardiovascular diseases, and death (Houston, 2007, Adams et al., 2012). For example, in Bangladesh where half of the population was exposed to arsenic contaminated water, 9,100 deaths and 125,000 disability adjusted years (DALYs) were reported in 2001 due to arsenic-contaminated water (Mamtani et al., 2011).

A compilation of over two decades of toxic metals emission and contamination data by the Metrological Synthesizing Centre-East, in Russia, revealed that a total of 266,381 t of lead, 6,228 t of cadmium and 3,868 t of mercury were released to the environment in over 50 European countries from 1990 to 2009 (MSC-E, 2011). Moreover, the Environment Agency of England and Wales estimated in 2005 that about 300,000 ha of land, or an estimated 325,000 sites, were contaminated with a variety of pollutants, with heavy metals accounting for a high proportion (Ashworth et al., 2005). Furthermore, an average of over 250 new sites in the UK have been classified as contaminated each year since 2000 due to heavy metal deposition (CL:AIRE, 2011).

As the world continues to respond positively to the principles of sustainability and stringent legislation from the European Union is leading to reduced pollution emissions, there is an urgent need to, effectively, and sustainably detoxify metal contaminated environments in order to guarantee quality living at every level in ecosystems.

This Chapter reviews the significance of heavy metals as environmental contaminants and describes their behavior and speciation in a dynamic soil-bacteria-plant system. It also critically evaluates current state of the knowledge on use of plant in toxic metal remediation and describes roles of plant growth promoting bacteria (PGPB) in phytoremediation.

## 2.2. Heavy metals: definition and properties

Although the use of the term ‘’heavy metals’’ to describe a subset of elements that exhibits metallic properties has been fiercely debated to be inappropriate (Duffus, 2002, Hodson, 2004), it is being widely used to refer to a group of metals and metalloids that are associated with environmental pollution and biological toxicity (Purakayastha and Chhonkar, 2010, Nagajyoti et al., 2010, Wang and Zang, 2014). Heavy metals has been defined as elements that exhibit metallic properties such as ductility, conductivity, cation stability, ligand specificity and have an atomic number that is greater than 20 (Naees et al., 2011). They are also described as elements with atomic density greater than 4 g $cm^{-3}$, or 5 times or more greater than that of water (Hawkes, 1997).

Apart from distinguishing heavy metals on the basis of their physical properties, chemical classification based on Lewis acids behavior and the ability of metals to

form complexes has been strongly advocated (Appenroth, 2010, Hodson, 2004). As Lewis acids, heavy metals are elements with a reactive vacant orbital or an available lowest unoccupied molecular orbital (Duffus, 2002).

The Department for Environment, Food and Rural Affairs (Defra) in the UK produced a list of elements regarded as heavy metals, and of which toxicities are monitored in the UK. These are: aluminum (Al), antimony (Sb), arsenic (As), barium (Ba), cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), iron (Fe), nickel (Ni), lead (Pb), manganese (Mn), molybdenum (Mo), rubidium (Rb), scandium (Sc), selenium (Se), strontium (Sr), Tin (Sn), titanium (Ti), tungsten (W), vanadium (V), and zinc (Zn) (Defra, 2008).

### 2.3. Sources and magnitude of heavy metal contamination

Apart from geogenic processes such as volcanic eruptions and rock weathering, anthropogenic activities are the major sources of heavy metals to the environment. Although heavy metal pollution is often regarded as the product of modern industrialization, the anthropogenic release of heavy metals has been traced to the beginning of the domestication of fire (Nriagu, 1996). Nevertheless, the more recent industrial production of textiles, plastics, wood preservatives, batteries and microelectronics (Ma et al., 2011a), the mining and smelting of metalliferous ores (Li et al., 2005a), the use of inorganic fertilizers and agrochemicals (Nicholson et al., 2003), the mass generation of sewage sludge and domestic waste (Purakayastha and Chhonkar, 2010), and the combustion of coal and fossil fuel (Reddy et al., 2005), are the major sources of heavy metal contamination in the environment.

It has been identified that a significant proportion of heavy metal deposition is from non-point sources through atmospheric fall out (Purakayastha and Chhonkar, 2010). Heavy metal emission from automotive emission, industrial plants and municipal waste combustion has been studied and well documented (Hasselriis and Licata, 1996, Lough et al., 2004, Reddy et al., 2005, Zechmeister et al., 2006).

Within the UK, an estimated 8, 259 t  Pb, 174 t Cd and 291 t Hg were emitted and from 1990 to 2009 (MSC-E, 2011). Pollutant maps show heavy metal deposition over the UK (Figure 2.1). The maps were generated by analyzing metal contents of rainwater collected at 15 sites and particulates collected through a network of Partisol 2025 sequential particulate samplers at 11 monitoring sites across the UK (Defra, 2010). It is however difficult to separate UK generated sources from cross border metal deposition. These maps are used to identify the areas where the metal deposition is most likely to cause a pollution effect (the red regions) as defined by the EC Framework Directive 96/62/EC that set out a common strategy to define and set objectives for ambient air quality.

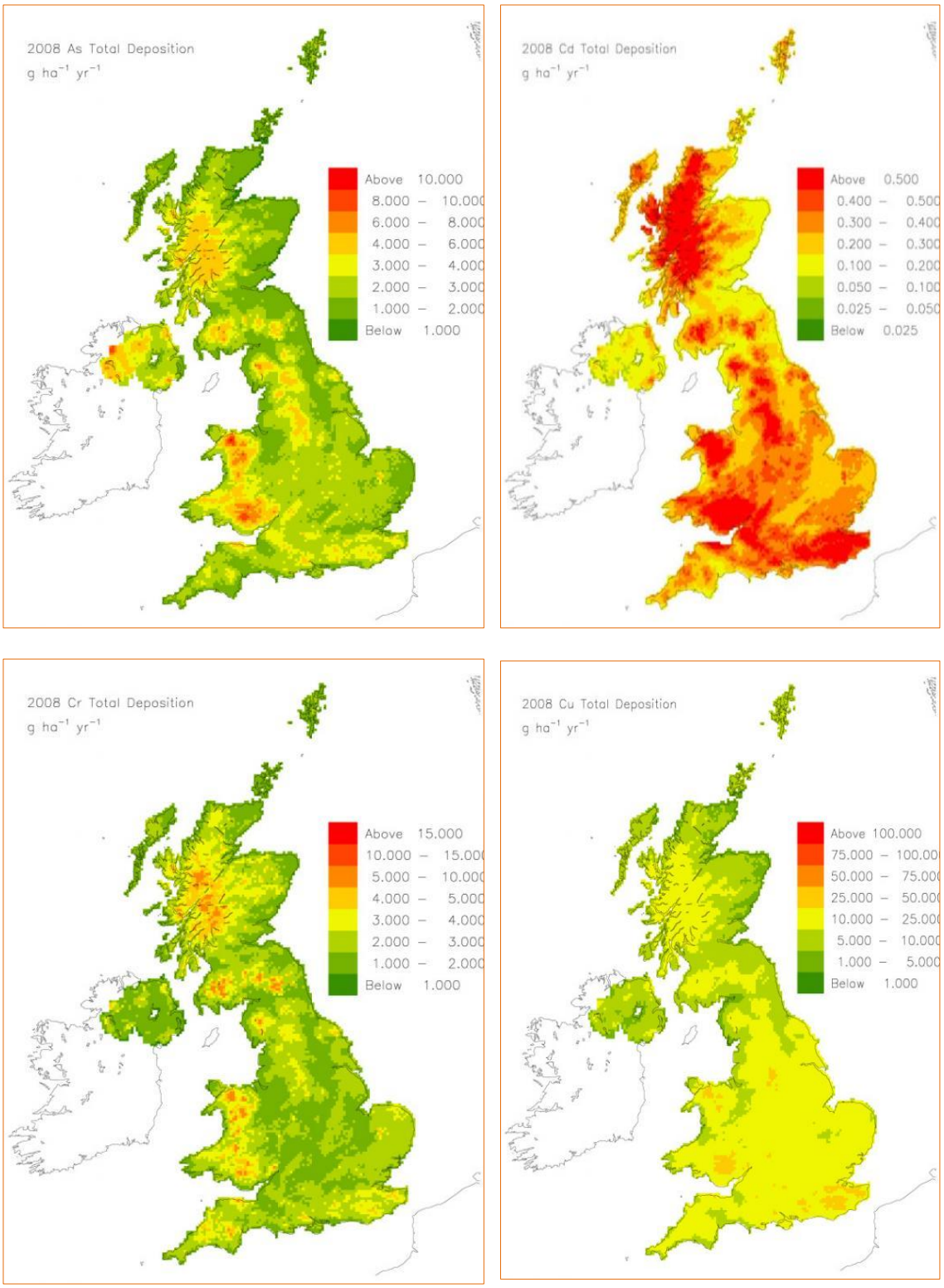



**Figure 2.1: Total As, Cd, Cr and Cu deposition in the UK in 2008 (Defra, 2010)**

Several attempts has been made to specifically characterise the spatial distribution and concentration of heavy metals in European soils to support the development of a EC Framework Directive that will set out common principles for the protection of soils across the EC (Chekushin et al., 2004, Imrie et al., 2008). The most comprehensive attempt to date involved modelling the concentrations of eight critical heavy metals in top soil of 26 European countries using 1,588 geo-referenced samples from the Forum of European Geological Surveys Geochemical database (Lado et al., 2008) (Figure 2.2).

Principal Component Analysis of the mapped heavy metals revealed that the units with the highest overall concentrations of heavy metals are: Huy and Liege (Belgium), Aachen (Germany), Attiki, Grevena and Kozani (Greece), and Coventry, Darlington and Hartlepool & Stockton (England). Moreover, in countries like England and Belgium heavy metal distributions and concentrations were suggested to be connected with the urbanization level, i.e. population density, while data from countries like Spain and the Czech Republic showed no obvious relationship to the level of urbanization (Lado et al., 2008). The results of metal contamination survey of (Lado et al., 2008) in European soils emphasis the significance of metals as important environmental contaminants and the needs for effective and sustainable metal remediation methods.

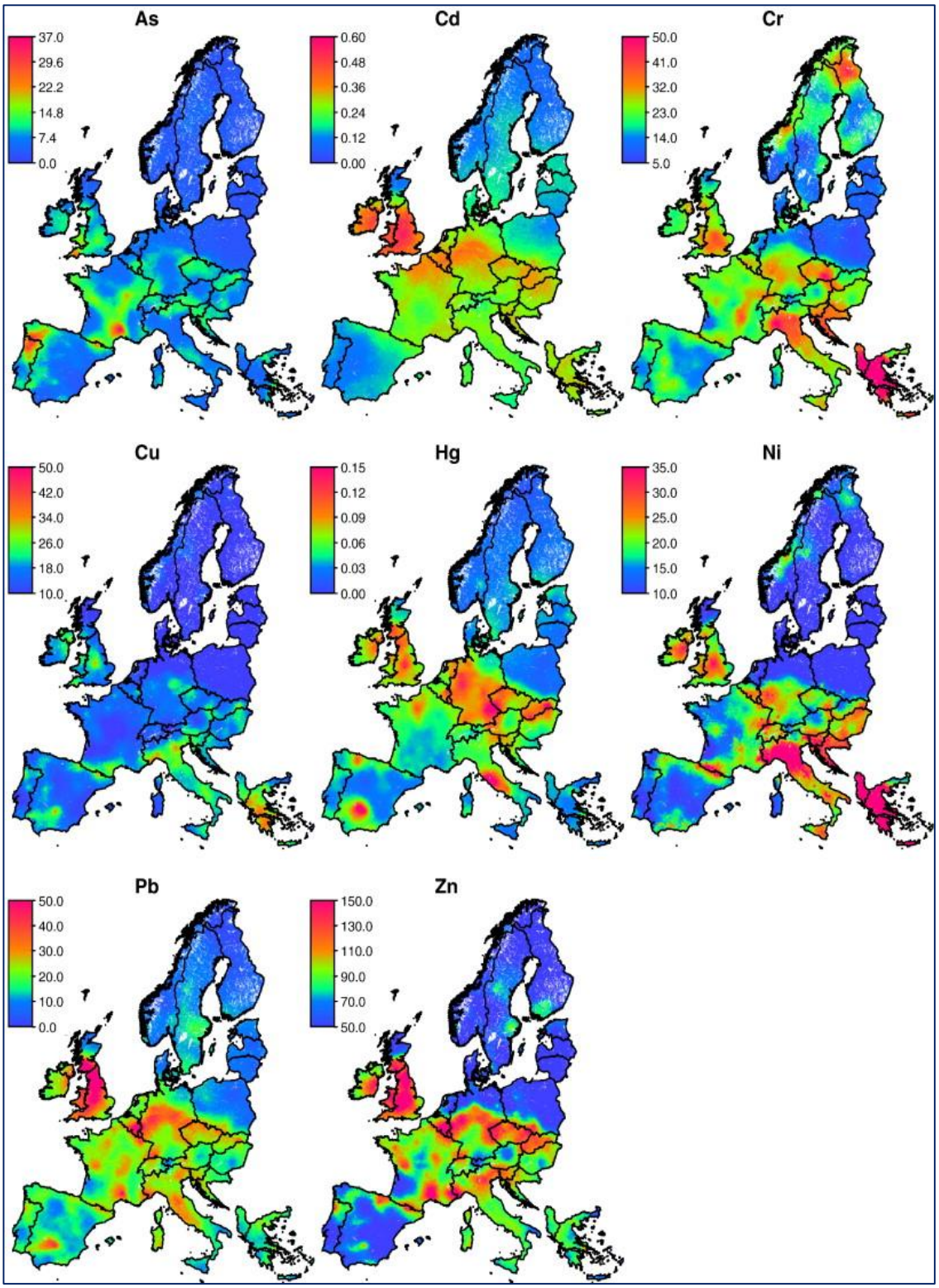



**Figure 2.2: Final maps of heavy metal concentrations in topsoil [mg $kg^{-1}$] interpolated using block regression-kriging (support size = 5 km) (Lado et al., 2008)**

### 2.4. Health and environmental concerns of metal contamination

Heavy metals are toxins that are capable of causing serious damage even at low concentrations (Duruibe et al., 2007, Nagajyoti et al., 2010). They are of serious concern to human health due to their cytotoxicity, mutagenicity and carcinogenicity (Järup, 2003, Lim and Schoenung, 2010). The ability of heavy metals to bio-accumulate in the human body, animals and in plants, increasing their chemical concentration over time within the biological system, makes them even more dangerous to human health (Kampa and Castanas, 2008, Cao et al., 2014). Although the specific number of deaths from heavy metal contamination have not been recorded, exposure to heavy metals has been discovered to damage or reduce the functioning of the mental and central nervous system, and significantly damage or lead to the complete failure of vital organs like the blood, lungs, kidney and the liver (Leung et al., 2010, García-Niño and Pedraza-Chaverrí, 2014). Long term exposure to heavy metals has also been found to result in neurological degenerative ailments such as Parkinson's disease, muscular dystrophy and multiple sclerosis (Kurlander and Patten, 1979, Gaggelli et al., 2012). Most importantly, contact with heavy metals has been reported to cause cancer (Türkdoğan et al., 2003, Zhao et al., 2014).

Symptoms of heavy metal toxicity in humans have been found to include cramping, nausea and vomiting, sweating, headaches, difficulty in breathing, impaired cognitive, motor, and language skills, mania, convulsions, learning difficulties, nervousness, emotional instability and insomnia (Graeme and Pollack, 1998, Tchounwou et al., 2012). Apart from these symptoms, toxic levels of heavy metals in humans are often assessed by measuring their concentrations in blood (Huo et al., 2007, Jin et al., 2014).

Ingestion through common hand-to-mouth activities is the major common route of exposure in children living in areas of toxic metal contamination. Moreover, children have been considered as a high risk group to heavy metal exposure due to the high likelihood of direct hand–to-mouth ingestion (Fels et al., 1998). In contrast, occupational exposure has been identified as one of the major routes for adults (Hengstler et al., 2003). Examples of high risk occupations are mining, industrial metal smelting, battery manufacturing, steel welding and rubber production (Mamtani et al., 2011).

The ability of heavy metals to enter the food chain has been identified as one of the most deadly threats to sustainable healthy living (Järup, 2003, Sridhara Chary et al., 2008). The translocation of heavy metals into edible crops grown in metal contaminated environments has been extensively studied and metal concentrations in the produce consumed have been found to exceed permissible dietary limits (Alloway et al., 1990, Alam et al., 2003, Lui et al., 2006, Celechovska et al., 2008). Furthermore, eco-toxicological studies have also revealed lethal doses of heavy metals in potable water (Wyatt et al., 1998, Roychowdhury et al., 2003) as well as in fish and fisheries products harvested from contaminated aquatic environments (Rashed, 2001, Mansour and Sidky, 2002).

The maximum permissible concentration (MPC) is the concentration of contaminants in the environment above which the risk of adverse effects is considered unacceptable to ecosystems (Crommentuijn et al., 2000). A comprehensive assessment of MPCs for 18 metals and metalloids in fresh water, ground water, soil

and sediments, which was used as the basis for formulating Environmental Quality Standards in the Netherlands, is presented in Table 2.1.

**Table 2.1: Maximum permissible concentration for metals and metalloids in fresh water, ground water, soil and sediment in the Netherlands (Crommentuijn et al., 2000)**

| Metal or metalloid | Fresh Water | Ground Water | Soil | Sediment |
|---|---|---|---|---|
| | Dissolved concentration in $\mu g\ l^{-1}$ | | $mg\ kg^{-1}$ standard soil or sediment which contain 10% organic matter and 25 % clay | |
| Antimony | 6.5 | 6.3 | 3.5 | 19 |
| Arsenic | 25 | 31 | 34 | 190 |
| Barium | 220 | 350 | 165 | 300 |
| Beryllium | 0.18 | 0.21 | 1.1 | 1.2 |
| Cadmium | 0.42 | 0.40 | 1.6 | 30 |
| Chromium | 8.7 | 11 | 100 | 1720 |
| Cobalt | 2.8 | 3.2 | 33 | 19 |
| Copper | 1.5 | 2.4 | 40 | 73 |
| Lead | 11 | 13 | 140 | 4,800 |
| Mercury | 0.24 | - | 2.2 | 26 |
| Methylmercury | 0.002 | - | 0.67 | 1.4 |
| Molybdenum | 290 | 290 | 254 | 250 |
| Nickel | 5.1 | 3.9 | 38 | 44 |
| Selenium | 5.3 | 5.3 | 0.81 | 2.9 |
| Thallium | 1.6 | 3.6 | 13 | 2.6 |
| Tin | 18 | 20 | 53 | 22,000 |
| Vanadium | 4.3 | 4.7 | 43 | 56 |
| Zinc | 9.4 | 31 | 160 | 620 |

The Codex Alimentarius Commission, a body that was established in 1961 by the Food and Agriculture Organization (FAO) of the United Nations and the World Health Organization (WHO) is responsible for protecting the health of food consumers and ensuring fair practices in the international food trade (Randell, 1995). The commission identified five metal contaminants to be of significant concerns to public health and established maximum permissible concentrations for these metals in edible commodities (Table 2.2).

**Table 2.2: Codex Alimentarius maximum permissible concentrations of metals in edible products (Commission, 1995) (The table shows the selected metals are toxic at very low concentrations)**

| Metal contaminant | Maximum permissible concentrations (mg $kg^{-1}$) | Edible commodities |
|---|---|---|
| Arsenic | 0.1 | Edible fats, Margarine, Edible vegetable oils, Olive oil |
| | 0.5 | Edible salt |
| | 0.01 (mg $l^{-1}$) | Natural mineral waters |
| Cadmium | 0.05 | Bulb vegetables, Fruiting vegetables |
| | 0.2 | Leafy vegetables |
| | 0.1 | Legume vegetables, Potato, Root and tuber vegetables |
| | 0.4 | Polished rice |
| | 0.5 | Edible salt, |
| | 0.003 (mg $l^{-1}$) | Natural mineral waters |
| Lead | 0.1 | Citrus fruits, Pome fruits, Fruiting vegetables, Bulb vegetables, Poultry meat, Edible fats, Margarine |
| | 0.2 | Berries and other small fruits, Legume vegetables, Pulses, |
| | 0.3 | Fish |
| | 0.5 | Edible offal of cattle, pig and poultry |
| | 2.0 | Edible salt |
| | 0.01 (mg $l^{-1}$) | Natural mineral water |
| Mercury | 0.1 | Edible salt |
| | 0.001 (mg $l^{-1}$) | Natural mineral waters |
| Tin | 50 | Meat; cooked cured and chop, Cooked cured ham and pork, |
| | 250 | Canned food and fruits |

## 2.5. Behaviour and speciation of metals in soil

Soil is the receptacle of all types of wastes and chemical elements (Korte et al., 1976, Essington, 2004), and the habitat for diverse micro and macro organisms (Wolters, 2001, Miyashita et al., 2013). The soil system is also the medium for plant growth, providing anchorage and supplying nutrients to plants (Miller and Donahue, 1990). Furthermore, the soil system influences surface water flows, and impacts on groundwater quantity and quality (Sliva and Dudley Williams, 2001). The physical and chemical properties of soils collectively play important roles in determining the activities and fate of metal contaminants (Vega et al., 2004, Sprynskyy et al., 2011). Understanding the behaviour (speciation, mobility and bioavailability) of heavy metals within the soil system is therefore crucial to the development of an effective bio-toxicity evaluation and sustainable remediation system.

### 2.5.1. Factors affecting metal behaviour in soils

Metal behavior in soils is affected by a number of factors and processes.

(i) Source/origin of metal contaminant: The origin of metal contaminants is important in assessing their behaviour in soils. For example, heavy metals that are native to the soil (pedogenic metals) are often found to be of low activity and largely immobile in the soil (Orrono and Lavado, 2009). This is in contrast with heavy metals from anthropogenic sources which have been found to be more active, mobile and more bioavailable to organisms (Chlopecka et al., 1996, Wang et al., 2006).

(ii) Soil texture: Apart from the influence of the source of metal contaminants on metal behavior, bioactivity of metal contaminants has been observed to be strongly correlated with soil texture (Temminghoff et al., 1997, Acosta et al., 2011). Generally, the finer soil particles (<2 μm diameter) exhibit higher adsorption ability for metal contaminants than the coarse fractions due to their increased specific surface area, higher clay minerals and organic matter content, and the presence of Fe–Mn oxides (Wang et al., 2006, Gong et al., 2014). Fine soil fractions are also mostly preferentially mobilised to the deeper soil layer and transported to surface/ground water (Gong et al., 2014). These properties make metals that are adsorbed to the fine soil fractions more difficult to remediate. Moreover, the finest soil particles of <10 μm diameter have been observed to be more readily re-suspended by air flows generated by wind or traffic to the atmosphere (Ho et al., 2003).

Adsorption efficiency of toxic metals to these fine soil fractions has been estimated to be 60-80% (Infante and Acosta, 1991). Inhaling these metal-enriched fine particles can therefore impair the respiratory system (Ajmone-Marsan et al., 2008). Soil fine particles, with a modal size of 39 μm diameter, adhere more to the hands of children increasing the potential for transfer to the mouth for ingestion (Yamamoto et al., 2006). With an estimate of 37 to 207 mg $day^{-1}$ daily ingestion rate of fine soil particles by children (Davis and Mirick, 2006), the risk posed by metal-enriched fine soil particles becomes more significant than the coarse fractions of the soil (Ajmone-Marsan et al., 2008).

(iii) Chemical properties of soil: Soil chemical properties also strongly influence the behaviour of metals in soils. Soil pH has been identified as the most important soil parameter influencing metal-solution and soil-surface chemistry (Harter, 1983, Heike, 2004, Akbulut et al., 2013). It is also the principal factor that governs the concentrations of soluble and bioavailable metals (Brallier et al., 1996, Houben et al., 2013). The soil system is largely made up of chemically charged soil colloids with high surface area and many adsorption sites (Karathanasis, 1999, Heike, 2004, Martin et al., 2014). Cation exchange of metals between the soil solids and release into the soil solution is dependent on the density of negative charges on the surfaces of soil colloids and the relative charges of metal species in solution (Eriksson, 1989, Rieuwerts et al., 1998, Bin et al., 2011).

Although the negative charges on clay minerals may be permanent, for example where isomorphous substitution of $Si^{4+}$ by $Al^{3+}$ has occurred, the occurrence of negative charges may also be pH dependent (Evans, 1989, Kosmulski, 2011). As soil pH increases the number of negatively charged surfaces also increases. The degree of metal adsorption to negatively charged surfaces in soils therefore significantly depends on soil solution pH. In general, metal solubility and biotoxicity increase at lower pH and decrease at higher pH values (Masscheleyn et al., 1991, Franklin et al., 2000, Heike, 2004, Heggelund et al., 2014). The adsorption affinities of heavy metals in relation to different adsorption sites and soil properties have been extensively studied (Swift et al., 1991, Dube et al., 2001). The most frequent heavy-metal cation selectivity sequence in five different soil types was found to be Cr>Pb>Cu>Cd>Zn>Ni> and Pb>Cr>Cu>Cd>Ni>Zn (Gomes et al., 2001).

(iv) Metal complexation with organic and inorganic ligands: Metal complexation with one or more organic or inorganic ligands has also been identified as an important influence on the biochemical behaviour of metals in the soil system (Rieuwerts et al., 1998, Violante et al., 2010, Park et al., 2011). Complexation of up to 99% of metals in soil solution has been reported in some Scottish soils (Berrow and Mitchell, 1980). The organic ligands involved in soil metal complexation include citric, gallic, oxalic acids and more complex acids contained in the soluble acid fractions of fulvic and humic acids (Evans, 1989, Rieuwerts et al., 1998, Parisová et al., 2013). Hydroxide and chloride ions are the important inorganic ligands involved in complexation of metals in soil solution (Garcia-Miragaya and Page, 1976, Rieuwerts et al., 1998, Sunda and Huntsman, 1998).

Apart from adsorption and complexation reactions, metal precipitation with anions like phosphate, carbonate and sulphate are also important reactions that influence metal bioavailability in soils (Patterson et al., 1977, McGowen et al., 2001, Márquez-Reyes et al., 2013). Metals in soil solution may also be precipitated as metal hydroxides, removing $OH^-$ ions from the soil solution (Basta and Tabatabai, 1992, Meunier et al., 2006). However, metal precipitation is mostly unlikely to occur in acidic soil conditions (Rieuwerts et al., 1998).

Strongly adsorbed, complexed or precipitated heavy metals have relatively reduced biochemical activity and eco-toxicity. However, metals held in an exchangeable form can later become available in soil solution as well as for plant absorption, depending on the prevailing soil chemical properties. Conceptual and quantitative models that take into consideration the nature of binding forces and variations of important soil

parameters have been developed to predict the fate and transportation of heavy metals within the soil-water-plant system (Heike, 2004).

### 2.5.2. Determining speciation of metals in soil

It is widely acknowledged that the mobility and toxicity of metal contaminants depends strongly on their specific chemical forms and on their binding states (Gleyzes et al., 2002, Marguı et al., 2004, Gu et al., 2014). Although quantification of total amounts of heavy metals in a soil is very important in risk assessment, quantification and analysis of metal speciation in relation to their adsorption, complexation or precipitation characteristics in soils gives a better indication of metal bioavailability and eco-toxicity (Gleyzes et al., 2002, Gu et al., 2014). Various extraction methods have been developed to quantify the different fractions or forms of heavy metal contents in soils (Hass and Fine, 2010, Trajković et al., 2014).

One of the most recognized methods is the European Community Bureau of Reference (BCR) sequential extraction method (Whalley and Grant, 1994, Davidson et al., 1998, Rauret et al., 1999). The procedure provides detailed information concerning the origin, mode of occurrence, biological and physico-chemical availabilities, mobilisation and transport of metals by simulating mobilisation and retention of metal species in the natural environment using changes in soil conditions such as pH, redox potential and degradation of organic matter (Passos et al., 2010). The method utilise acetic acid (0.11 mol $L^{-1}$), hydroxylamine hydrochloride (0.5 mol $L^{-1}$), hydrogen peroxide (8.8 ml $L^{-1}$) and ammonium acetate (1.0 mol $L^{-1}$) to extract exchangeable (acid soluble and traditionally considered as the bioavailable fraction),

reducible (metals associated with oxides of Fe and Mn) and oxidisable (metals associated with organic matter and sulphides) fractions in 1 g of soil sample (Rauret et al., 1999, Passos et al., 2010). An additional fourth fraction that is often strongly adsorbed to the soil minerals (residual metal contents) is often extracted in aqua regia; a 1:3 mixture of concentrated nitric and hydrochloric acids (Relić et al., 2011).

Metal speciation in a broader sense, is defined as the distribution of metals among their various chemical and physical forms, and possibly oxidation states (Parker et al., 1995). This includes the description of the metal free ions, complexes, ion pairs, chelates in soil solution, and amorphous and crystalline solid-phases (Gräfe et al., 2014). These characteristics that define the speciation of a metal collectively influence metal reactivity, mobility and bioavailability and toxicity (Adamu et al., 2013, Gräfe et al., 2014). The use of sequential extraction is therefore not sufficient in providing adequate information about metal speciation in soils. Apart from possible inaccuracy from dissolution of non-target phases, incomplete dissolution of a target phase, re-adsorption or re-precipitation leading to partial capture of dissolved species, and possible modification of the original oxidation states of metal, sequential extraction methods cannot provide information about the metal's crystalline structure and may therefore not reflect the exact chemical status and nature of metals in the contaminated environment (Ostergren et al., 1999, Scheinost et al., 2002, Kirpichtchikova et al., 2006).

The advent of synchrotron based X-ray Absorption Spectroscopy (XAS) techniques has revolutionised the study of metal speciation in the past 20 years. XAS is used to

investigate metal speciation in both biological and environmental samples because of the ability of the technique to characterize both amorphous and crystalline properties (Parsons et al., 2002). XAS measurement can be described in two basic steps:

(i) When a sample is bombarded with X-rays of a specific energy, electrons absorb the energy until they are ejected from a particular bidding shell in an atom of the sample and (ii) the oscillation of the displaced electrons reduces as absorbed energy dissipates (Parsons et al., 2002). Because bound electrons have well-defined binding energies that can be quantified (Powell, 1995, Elam et al., 2002), the changes in energy during electron ejection and subsequent oscillations can be measured to describe the nature of the sample (Parsons et al., 2002).

XAS data collection therefore consists of two different but complimentary measurements of: (i) X-ray absorption near edge structure (XANES) and (ii) extended X-ray absorption fine structure (EXAFS) (Parsons et al., 2002). XANES provides information on the oxidation state, three-dimensional geometry, and coordination environment of the metal under investigation and EXAFS provides information on the coordination environment and nearest neighbouring atoms to the atom of interest (Parsons et al., 2002).

A depiction of XAS experiments and the XANES and EXAFS regions of a XAS spectrum are presented in Figure 2.3.

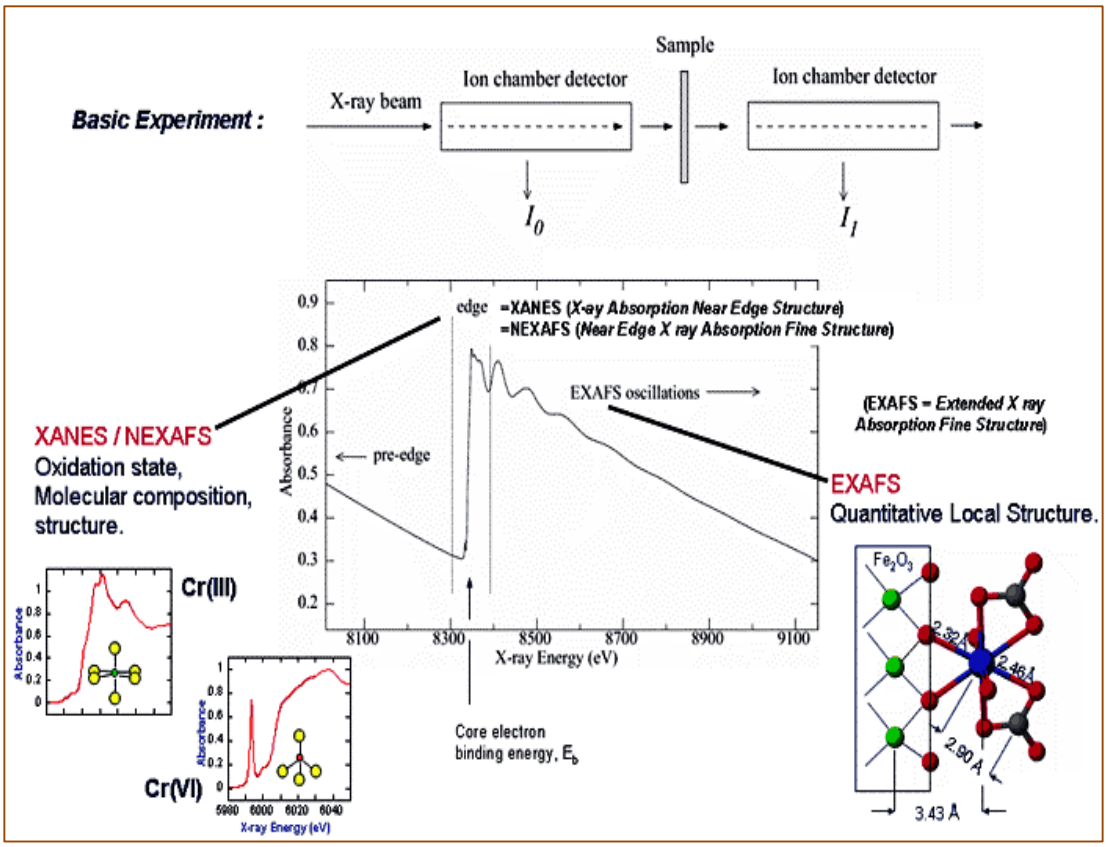



**Figure 2.3: An illustration of basic X-Ray Absorption Spectroscopy (XAS) and the types of information obtained from XAS (SSRL, 2009)**

A detailed description of the use of XAS data collection and modelling in this research is presented in Chapter 3.

### 2.5.3 Mechanisms of metal uptake by plants

Plants are very important components of the ecosystem because of their ability to transform abiotic elements from the environment to biotic plant components in significant amounts (Chojnacka et al., 2005). The three main mechanisms of metal uptake in plants are: (i) contact (surface) exchange between plant roots and exchangeable metals, (ii) diffusion of metals from the region of high concentrated solution (the bulk soil) to a region of low concentrations (the rhizosphere) and (iii) the mass movement of dissolved metals in soil solution under the influence of transpiration pull (Rao and Mathur, 1994, Planquart et al., 1999). The mobilization of

the colloidal bound heavy metals and their subsequent solubilisation is often achieved through proton extrusion from the root, secretion of metal-chelating molecules and the release of specific plasma-membrane bound reductase by plants (Salt et al., 1995). Secretion of phytosiderophores for metal chelation has also been found to aid desorption and solubilisation of metals in soils (Salt et al., 1995).

Once the absorbed heavy metals become bioavailable, they can be translocated into the plant system through symplastic (intercellular uptake against an energy gradient) or apoplastic (extracellular absorption through a permeable membrane) mechanisms (Ghosh and Singh, 2005b) depending on the types and concentrations of metals and plant species (Jabeen et al., 2009). The absorbed heavy metals can then be stored within the plant roots or translocated through the xylem to the shoots, leaves or fruits (Jabeen et al., 2009). Some heavy metals, such as Cu, Mn, Mo, Ni and Zn, are essential plant nutrients when available in minute concentration (5-100 mg $kg^{-1}$ DM) to plants (Alloway, 2013). These elements however become phytotoxic as soon as the essential concentrations are exceeded (Alloway, 2013).

### 2.5.4. Mechanisms of metal toxicity and tolerance in plants

Mechanisms of metal toxicity have been researched in many plant species (Foy et al., 1978, Schützendübel and Polle, 2002). Induction of oxidative stress through the production of reactive oxygen species (ROS) has been identified as a major response reaction in plants exposed to high levels of heavy metals (Yadav, 2010, DalCorso, 2012). This involves the successive reduction of molecular oxygen to reactive oxygen species such as $H_2O_2$, $OH^{\cdot}$ & $O_2^{\cdot -}$ which may lead to oxidation of proteins,

membrane lipids or DNA damage to plant cells (Schützendübel and Polle, 2002). Plant tissues that are injured by metal induced oxidative stress often contain increased concentrations of carbonylated proteins, malondialdehyde and deleterious levels of ethylene (Dean et al., 1993, Schützendübel and Polle, 2002).

Furthermore, the binding of heavy metals to the sulfhydryl functional groups (–SH) of structural plant proteins and enzymes (Schützendübel and Polle, 2002) has been demonstrated to prevent correct DNA folding, interfere with catalytic reactions, and perturb enzyme-mediated redox regulation, subsequently leading to damage and death of plant cells (Hall, 2002, DalCorso, 2012). Moreover, the displacement of essential ionic cofactors in vital plant enzymes and signalling proteins by heavy metal ions has been observed to cause loss of cellular biochemical activity and perturbation of gene expression in plant cells (DalCorso, 2012).
The heavy metal induced displacement reactions can also interfere with homeostatic pathways for obtaining essential plant nutrients (Roth et al., 2006).

Seed germination is the first physiological process to be affected by toxic metals (Shah et al., 2010) and evaluation of seed germination in media contaminated with various doses of metals is often used to assess metal tolerance in plants (Kranner and Colville, 2011). Roots are the first plant organs to come into contact with toxic metals and usually accumulate more metals than any other plant organ (Shah et al., 2010, DalCorso et al., 2013). Impairment of root growth is therefore often the first visible symptom of toxic metal uptake in plants (Shah et al., 2010) and root length measurements can be used as an index of the tolerance of plants to metal

contamination (Belimov et al., 2003, Leung, 2013). Shoot growth, as well as plant height, is also hindered by high metal concentrations, possibly due to reduced root growth and lower transport of essential nutrients and water to aerial plant parts (Shah et al., 2010, Sinha et al., 2010). Necrosis of younger leaves and drying and chlorosis of older leaves are other visible symptoms of heavy metal toxicity in many plant species (Pandey and Sharma, 2002, Yadav, 2010).

However, some plant species have developed potential mechanism(s) for surviving metal toxicity in heavy metal contaminated environments. A significant volume of research has been dedicated to identifying the specific mechanism(s) through which plants survive metal toxicity, but no specific mechanism has yet gained global recognition or acceptance. Nevertheless, survival of metal toxicity by plants is broadly divided into two main classes of process: avoidance and tolerance (Baker, 1987, Rascio and Navari-Izzo, 2011).

Survival of metal toxicity through avoidance occurs in which the plant is protected externally from the influence of metal induced toxicity (Baker, 1987). The main avoidance process involves prevention of metal uptake into root cells through entrapment of metals in an apoplastic environment by binding them to exuded organic acids or to ionic groups on plant cell walls (Rascio and Navari-Izzo, 2011). For example, the complexation of metals with pectic acid (also known as polygalacturonic) secreted at the cell wall of plants has been shown to reduce metal toxicity to plants (Cataldo et al., 2012, Dalvi and Bhalerao, 2013).

This restricts metal translocation to above ground plant biomass, protecting leaf tissues and especially photosynthetic cells from toxicity damage (Rascio and Navari-Izzo, 2011).

In contrast, surviving metal toxicity through tolerance involves physiological processes which enable the plant to function in the presence of high concentrations of heavy metals (Baker, 1987). Metal tolerant plants are capable of accumulating large amounts of metals in their biomass (above and below ground) due to the production of metal binding compounds, compartmentalisation of metals in less sensitive organs and secretion of antioxidants which counteract metal induced oxidative stress (Baker et al., 1994a, Shah et al., 2010). The binding of metal with metallothioneins and phytochelatins like phytate, cysteine, and histidine in plants has been reported to play major roles in metal detoxification and homeostasis (Meharg, 1994, Cobbett and Goldsbrough, 2002, Gupta et al., 2013).

The differences in inherent response mechanisms to metal contamination in plants have been explored and some plant species have been classified as metal excluders and metal (phyto) remediators, which are further sub-divided into phytostabilisers and hyperaccumulators (Baker, 1981, Rascio and Navari-Izzo, 2011).

The digestion of oven dried or ashed plant biomass with concentrated hydrochloric, sulphuric, nitric or perchloric acids and analysis of digests by atomic absorption spectrometry (AAS) or inductively coupled plasma (ICP) spectrometry are widely-used methods of evaluating metal concentrations in plants (Lowther, 1980, Hseu, 2004). Although this analytical method is good for measuring total metal

accumulation in plant biomass, it's however destructive and does not allow investigation of forms of metal storage and speciation in fresh plant organs. In this research synchrotron based XAS was utilised to provide insight into tolerance mechanisms in plants exposed to high metal concentrations. A detailed description of the method is presented in Chapter 3 and results from the analysis are presented in Chapters 4, 5, 6 and 7.

### 2.5.5. Metal toxicity and tolerance in microorganisms

Microorganisms are a major component of metal contaminated environment as they have been reported to be capable of influencing the behaviour of metals, promote plant growth under metal toxicity and provide valuable indications for monitoring metal pollution (Brookes, 1995, White et al., 1995, Khan, 2005). Toxicity of metals to soil and plant associated microorganisms has been widely studied in many species under various experimental conditions (Giller et al., 1998, Wang et al., 2007).

The main components of the soil microbial community are the fungi and bacteria (Rajapaksha et al., 2004). They may exist as free-living fungi or bacteria, or as symbiotic mycorrhizal fungi or rhizobacteria with plants (Khan, 2005).

Fungi are often regarded as more tolerant to metal toxicity than bacteria (Hiroki, 1992, Rajapaksha et al., 2004). Metal tolerance mechanisms in fungi include: extracellular precipitation, chelation and binding of metals to cell walls; intracellular chelation of metals with organic acids, polyphosphates, peptides; and transport of metals into intracellular compartments (Bellion et al., 2006, Cuypers et al., 2013). Mycorrhizal fungi have been shown to enhance metal tolerance in their host plants

(Adriaensen et al., 2006, Redon et al., 2009). Apart from possible influences on metal homeostasis and detoxification, mycorrhizal fungi can improve nutrition of the host plant due to the ability of the external fungal hyphae to exploit a larger proportion of nutrient resources in the contaminated soil (Khan, 2005).

Arbuscular mycorrhizal fungi often recognize and associate with the host plant through root exudates secreted by the plant (Giovannetti et al., 1994, Khan, 2005) which stimulate spore germination and growth of mycorrhizal hyphae (Khan, 2005). However, non-mycorrhizal host plants such as mustard, spinach, sugar beet and lupin secrete exudates that inhibit spore germination and reduce root colonisation by fungi (Oba et al., 2002, Khan, 2005). Moreover, fungi such as *Pythium mamillatum* and *Pythium ultimum* have been reported to be pathogenic to plants, causing damping-off disease in seedlings of *Alyssum serpyllifolium* and *Alyssum murale* both of which are metal hyperaccumulating plant (Ghaderian et al., 2000). Therefore the use of fungi is limited in enhancing the tolerance of plants for remediating metal contaminated soils.

Metal tolerance in many species of bacteria has been observed to be controlled by the bacterial plasmids which carry the genes for resistance to many metals (Trevors et al., 1985, Silver and Phung, 1996, Gutiérrez-Barranquero et al., 2013). Although the specific mechanism behind metal tolerance in bacteria is yet to be identified, efflux of metal ions outside the bacterial cell wall, sequestration of metallothioneins and reduction of metal ions to less toxic forms have been demonstrated as possible mechanisms for bacterial survival under metal toxicity (Choudhury and Srivastava, 2001, Saluja and Sharma, 2013).

Some species of bacteria have been identified to promote plant growth and they are generally referred to as plant growth promoting bacteria (PGPB) (Bashan, 1998, Compant et al., 2005). Addition of metal tolerant PGPB into the plant-contaminant system through plant or soil inoculation has been widely reported to significantly promote the growth of hyperaccumulators exposed to contaminants (Burd et al., 2000, Glick, 2003, Rojas-Tapias et al., 2012).

The specific mechanism through which PGPB promote plant growth under heavy metal contamination is still unknown. Many species of PGPB have been identified (Belimov et al., 2005, Goes et al., 2009) but none has yet gained acceptance as the most efficient in microbial-phytoremediation of heavy metal contaminated soils.

### 2.6. Bioremediation of heavy metal contaminated soils

Although heavy metals cannot be biologically destroyed, their ability to be transformed from one oxidation state to another and/or be changed from an organically complexed state to another (Garbisu and Alkorta, 2001, Gavrilescu, 2004, Mateos et al., 2010) is being explored to remediate heavy metal contaminated environments. Mechanical and chemical methods that are used for remediating metal contaminated sites include excavation and landfilling of contaminated soil, thermal treatment, acid leaching and electro-reclamation (Mulligan et al., 2001b, Jing et al., 2007, Zhang et al., 2013a). However, these methods are extremely expensive and have been declared as not economically viable in most situations (Mulligan et al., 2001b, Purakayastha and Chhonkar, 2010). For example, it is estimated that in order to reduce soil Pb concentration from 1.4 g/kg to 0.4 g/kg in ten years,

phytoremediation will cost only $27,900 while landfilling and soil leaching methods will cost $1,620,000 and $790,000 respectively (Wu et al., 2010).

Apart from their high costs, these methods have been found to be dependent on contaminant and site properties (Jing et al., 2007), permanently destroy soil structure and fertility (McGrath et al., 1995), destabilise the natural ecosystem and are aesthetically unpleasant (Purakayastha and Chhonkar, 2010, Wu et al., 2010). Therefore, an environmentally preferable, affordable, efficient and aesthetically pleasing remediation method has been sought.

Bioremediation has been defined as a process of biologically degrading waste under controlled conditions to levels below permissible environmental limits established by regulatory authorities (Mueller et al., 1996). It has also been defined as 'technology that uses metabolic processes to degrade or transform contaminants, so that they remain no longer in harmful form' (Ramasamy et al., 2007). Although, the word 'bioremediation' has been used by many researchers to refer to the use of the metabolic functions of microorganisms alone to remediate contaminated environment (Sayler and Ripp, 2000, Iwamoto and Nasu, 2001, Kazuya, 2001), modern definitions now include plant and/or microorganisms as biological agents for decontamination (Shukla et al., 2010, Akhtar et al., 2013b). Mechanisms of bioremediation and phytoremediation are described in detail below.

### 2.6.1. Microbial bioremediation

Microorganisms are one the most abundant forms of life in the geosphere. They are ubiquitous, possess unique abilities to survive in hostile environments and are able to transform all forms of minerals and organic materials (Bollag et al., 1994,

Fredrickson and Onstott, 1996). Of all microorganisms, bacteria clearly stand out for their population in un-contaminated and contaminated environments and for the enormous surface area they present for biochemical reactions in the earth's crust (Fein et al., 1997). An estimated $10^6$-$10^{10}$ bacteria cells are reported to be present in 1 g of soil, rock or sediment (Barns and Nierzwicki-Bauer, 1997).

The mechanisms of bacterial remediation have been classified into three major processes of bio-sorption, bioaccumulation, and enzymatic reduction and immobilization (Ramasamy et al., 2007).

(i) *Bio-sorption*

Metal adsorption by bacteria is possible due to the ionic nature of their cell wall (Fein et al., 1997, Ngwenya et al., 2003, Vijayaraghavan and Yun, 2008). The cell walls of bacteria have been reported to possess several organic functional groups, such as the hydroxyl, carboxyl, phosphate and amino functional groups (Beveridge and Murray, 1980, Jiang et al., 2004, Liu et al., 2013). These organic functional present negative charges for metal adsorption due to their deprotonation under pH conditions greater than 2 (Fein, 2000, Liu et al., 2013). The phenomenon of metal adsorption to bacterial cell wall has been described in terms of equilibrium thermodynamics under varying chemical conditions (especially pH) (Fein et al., 1997, Paul et al., 2012), and surface complexation models (with adjustable parameters) have been used to quantify the extent and thermodynamics of metal adsorption (Fowle et al., 2000, Ngwenya et al., 2003, Cox et al., 1999, Gorman-Lewis et al., 2014).

*(ii) Bioaccumulation*

The kinetics of bioaccumulation involves the active translocation of heavy metals across the cell membrane of bacteria into the cell cytoplasm (Anushree, 2004,

Ramasamy et al., 2007). This metabolic absorption has been reported to mimic the same process as essential nutrients uptake in bacteria (Veglio and Beolchini, 1997). However, both passive adsorption and metabolic uptake have been regarded by some researchers as a single biphasic metal uptake process; involving an initial rapid but passive adsorption of metals to the bacterial surface, and subsequent slower but active absorption of adsorbed metals into the microbial cells (Garnham et al., 1992, Dönmez and Aksu, 1999). A metal that has been translocated into the cell cytoplasm of bacteria is immobile, and therefore regarded to be sequestrated (Losi et al., 1994, Mishra and Malik, 2013).

*(iii) Enzymatic reduction and immobilization*

Most heavy metals in their lowest oxidation state are soluble, readily mobilized into groundwater and thus toxic to living tissues in the environment. However, some species of bacteria are capable of changing the redox potential of heavy metal contaminated environments and thereby rendering a soluble metal into an insoluble form (Michel et al., 2001, Ramasamy et al., 2007). Iron, manganese and sulphate reducing bacteria have been reported to be capable of immobilising heavy metals. These bacteria first reduce $Fe^{3+}$, $Mn^{4+}$ and $SO_4^{2-}$ causing the release of Fe(II), Mn(II) and hydrogen sulphide respectively to the environment (Lovley et al., 1989, Tebo and Obraztsova, 1998, Thamdrup, 2000). The released reduced forms then react chemically with soluble oxidised heavy metals to immobilise the heavy metals into soluble sequestrated metals (Roh et al., 2006, Ramasamy et al., 2007). Conversely, the reduction of oxidised heavy metals may also occur through a process mediated by microbial-secreted enzymes (Lovley et al., 1993, Silver and Phung, 2005).

#### 2.6.1.1. Challenges in microbial bioremediation

Despite the benefits of microbial bioremediation as a low cost and non-invasive bioremediation technique, it is only effective where environmental conditions permit microbial growth and activity (Vidali, 2001). The use of metal tolerant microorganisms that have adapted to the organic matter content, temperature, pH, and redox potential of the contaminated site has been suggested, but most microorganisms appear to be capable of remediating a specific metal contaminant (Vidali, 2001, Tabak et al., 2005). Moreover, some metals are resistant to microbial remediation and the bioremediation rates of biodegradable metals are very slow (Boopathy, 2000, Vidali, 2001).

### 2.6.2. Phytoremediation

Plants on the other hand, are natural miners of minerals in the Earth's crust, a characteristic that is being explored to develop a "green" remediation technology. Phytoremediation (coined from the Greek noun, *phyton*, and the Latin verb, *remediare*) (Sinha et al., 2007) is a low energy, low cost and environmentally preferable bioengineering process that makes use of green plants to sequester contaminants from contaminated environments (Chappell, 1997, Pilon-Smits, 2005, Jabeen et al., 2009, Glick, 2010, Hamidian et al., 2014).

A number of aquatic and terrestrial plants, including grasses, herbs, shrubs and trees, have been discovered to have a high tolerance for water and toxicity stress and possess an excellent ability for significant extraction of non-metabolic elements from contaminated environments (Sinha et al., 2007, Muhammad et al., 2013, Manousaki et al., 2014). The biophysical and biochemical mechanisms that govern

phytoremediation have been extensively studied. Widely reported phytoremediation processes are briefly discussed below.

(i) *Phytoextraction and phytoaccumulation*

Phytoextraction and phytoaccumulation are elements of a process that results in the significant uptake of bioavailable contaminants from the soil by plant root systems and the subsequent accretion of the extracted elements into the above ground plant shoots, leaves and fruits (Eapen et al., 2007, Jabeen et al., 2009, Alyazouri et al., 2014). Plant species and genotypes that exhibit this unique characteristic are called hyper-accumulators, defined as plants with the capacity to translocate and accumulate more than 0.1% Pb, Co, Cr, or more than 1.0% Mn, Ni or Zn in their aerial parts (Baker and Brooks, 1989). Hyperaccumulator plants have also been defined as those with a shoot dry matter concentration of > 1000 mg $kg^{-1}$ Cu, > 5,000 mg $kg^{-1}$ Pb or > 10,000 mg $kg^{-1}$ Zn (Haque et al., 2007). Moreover, plants regarded as hyperaccumulators are expected to have above ground heavy metal concentrations 10 to 500 times greater than those of normal plants (Shen and Liu, 1998).

Apart from being a hyperaccumulator, an ideal plant for phytoremediation must be tolerant to high metal toxicity, should exhibit excellent ability for rapid growth even under various stress factors, must have high vegetative volume (biomass), and possess an effective profuse rooting system (Salt et al., 1995, Blaylock and Huang, 2000, Gisbert et al., 2003). A wide number of plants have been demonstrated to be hyperaccumulators of different types and amounts of metals. A compilation of hyperaccumulators, the types of metal they accumulate and the amount of metal they accumulates in their above ground biomass is presented in Table 2.3.

Table 2.3: Important plant hyperaccumulators of heavy metals (Purakayastha and Chhonkar, 2010)

| Metal | Hyperaccumulator | Reported concentration(mg $kg^{-1}$) | References |
|---|---|---|---|
| Zinc | *Thlaspi caerulescens* | 52,000 | Brown et al. (1994) |
| | *Streptanthus polygaloides* | 6,000 | Boyd and Davis (2001) |
| | *Potentialla griffithii* | 6,250 | Qiu et al. (2006) |
| Copper | *Ipomoea alpine* | 12,300 | Baker and Walker (1990) |
| | *S. polygaloides* | 120 | Boyd and Davis (2001) |
| | *Medicago sativa* | 85 | Videa-Peralta (2002) |
| | *Brassica juncea* | 22 | Purakayastha et al. (2008b) |
| Cadmium | *T. caerulescens* | 1,800 | Baker and Walker (1990) |
| | *Alfa alfa* | 1,079 | Videa-Peralta (2002) |
| | *Nicotiana tabacum* | 40 | Evangelou et al. (2004) |
| | *Sinapis alba* | 123 | Evangelou et al. (2007) |
| Lead | *Thlaspi rotundifolium* | 8,200 | Baker and Walker (1990) |
| | *Pisum sativum* | 8,960 | Huang et al. (1997) |
| | *B. juncea* | 15,000 | Blaylock et al. (1997) |
| | *T. caerulescens* | 844 | Robinson et al. (1998) |
| | *Vertiberia Zizanioides* | 1,450 | Wilde et al. (2005) |
| | *Sonchus arvensis* | 3,664 | Surat et al. (2008) |
| Nickel | *Sebertia acumunata* | 25% wt. of dried sap | Jaffre et al. (1976) |
| | *Alyssum lesbiacum* | 47,500 | Küpper et al. (2001) |
| | *Medicago sativa* | 437 | Videa-Peralta (2002) |
| | *Alyssum bracteatum* | 2,300 | Ghaderian et al. (2007) |
| Chromium | *Leptospermum scoparium* | 20,000 | Baker and Brooks (1989) |
| | *B. juncea* | 1,400 | Shahandeh and Hossner (2000) |
| | *Helianthus annus* | | Shahandeh and Hossner (2000) |
| Selenium | *Astragalus racemosus* | 14,900 | Beath et al. (1937) |
| | *Astragalus pectinalus* | 4,000 | Shrift (1969) |
| | *Stanleyea pinnola* | 330 | Shrift (1969) |
| | *Actiniopteris radiate* | 1,028 | Srivastava et al. (2005) |
| Arsenic | *Pteris vittata* | 23,000 | Ma et al. (2001) |
| | *Pityrogramma calomenalos* | 8,350 | Francesconi et al. (2002) |
| | *Pteris multifida* | 1,977 | Wang et al. (2006) |

The plant parts where toxic metals accumulate, can then be disposed of or their metal contents extracted for reuse (Purakayastha and Chhonkar, 2010, Ghosh and Singh, 2005a). A process of phytoextraction, accumulation and disposal or reuse of sequestrated toxic metals in plants is illustrated in Figure 2.4.

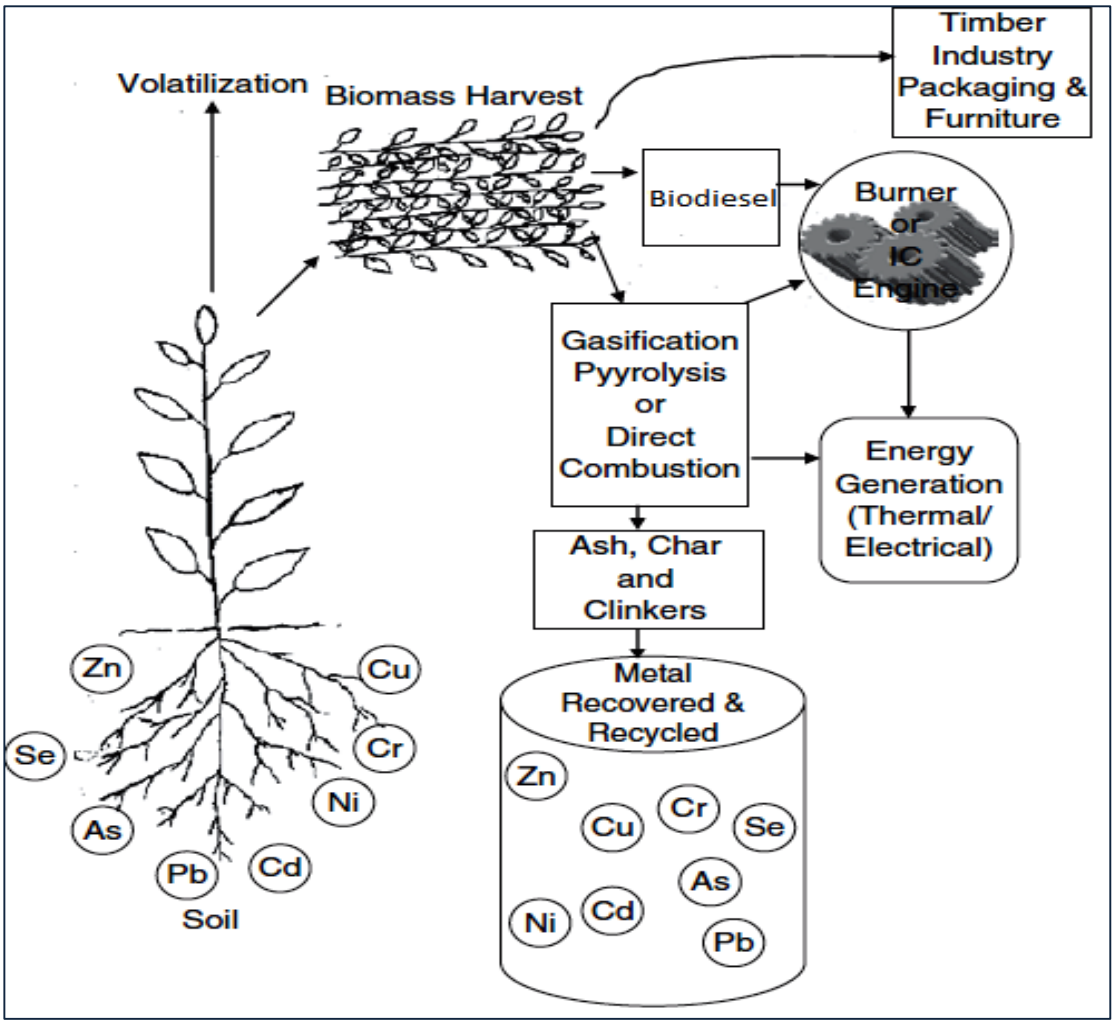



**Figure 2.4: Processes of phytoextraction, accumulation and recycling of plants used in metal phytoremediation (Purakayastha and Chhonkar, 2010)**

*(ii) Phytovolatization*

Phytovolatization involves the transformation of toxic species of some heavy metals (e.g. Hg) into volatile gases (less toxic species) through the process of transpiration (Salt et al., 1998, Sinha et al., 2007, Jabeen et al., 2009). The transpired heavy metals from contaminated soil and water move through the plant and are released to the atmosphere from the aerial part of the plant. The use of phytovolatization in sites contaminated by Hg and Se has been reported to be successful (Ghosh and Singh, 2005b). In most cases, phytovolatization is however regarded as part of phytoextraction.

*(iii) Phytostabilisation*

Phytostabilisation refers to the reaction or strong adsorption of charged heavy metals to plant roots, thus immobilizing heavy metals and significantly reducing their toxicity in the environment (Eapen and D'Souza, 2005, Dary et al., 2010, Galende et al., 2014). In contrast to hyperaccumulators where effective root to shoot element translocation is required, phytostabilisers are expected to be poor translocators of metals, exhibit high tolerance to environmental stress and have a dense root system (Eapen et al., 2007, Lee et al., 2014). Phytostabilisation is mostly used in-situ as a phytoremediation method for contaminated sites that are not under pressure to be used for other purposes, thus allowing time for the plants to fully establish on the contaminated site (Dary et al., 2010). For example, suppression of vertical migration of heavy metals in the soil by the action of the phytostabilisers was reported to significantly reduce the incidence of ground water pollution (Cunningham et al., 1995).

### 2.6.2.1. Challenges in phytoremediation

Slower rate of metal remediation because of slow rate of plant growth and reduced biomass under metal contamination is the main disadvantage of phytoremediation when compared to chemical or mechanical remediation methods (Salt et al., 1998, Lasat, 2000). Despite the inherent tolerance of metal remediating plants to metal toxicity, they may still suffer from adverse weather conditions, weeds, pests and diseases (Gerhardt et al., 2009). Moreover, plants may suffer nutrient deficiencies and hormonal imbalances induced by metal contamination (Belimov et al., 2005,

Maksymiec, 2007). These possible indirect metal toxicity effects in plants under metal contamination are discussed as follows.

*(i) Macronutrient deficiency*

Most metal contaminated soils have been found to be deficient in nitrogen, the nutrient required in large amounts by plant for vital metabolic functions, such as photosynthesis, and for optimum vegetative growth (Wong, 2003, Brown et al., 2003). This may be due to a negative impact of metal toxicity on nitrogen fixation and overall nitrogen metabolism by microorganisms in the soil (Singh et al., 2009, Gajewska and Skłodowska, 2009). Moreover, fixation of phosphates and reduced availability to plants has also been widely reported in heavy metal contaminated environments (Bolan et al., 2003, Rajkumar and Freitas, 2008). Phosphates mineralise to yield phosphorous, a macro element that is necessary for vital plant metabolic functions, especially the production of adenosine triphosphate (ATP) that provides the energy for all metabolic processes in plants (Zaidi et al., 2006). Phosphate adsorption to heavy metals is increased in low pH conditions (Chen et al., 1997, Aklil et al., 2004). The combined deficiency of these macro-elements in heavy metal contaminated environments is enough to cause complete wilting of heavy metal tolerant plants within a few weeks of planting in a contaminated soil (Lasat, 2000, Tripathi et al., 2014).

*(ii) Micronutrient deficiency*

Iron is often reported to be deficient in heavy metal contaminated soils, but is one of the essential nutrients for optimum photosynthesis in plants (Cakmak et al., 1994,

Glick, 2003). Iron deficiency will therefore lead to plant chlorosis and subsequent wilting of the plant (Ramírez et al., 2013). Antagonistic reaction between Fe and other heavy metal micronutrients such as Zn, where the heavy metal manifests itself for uptake at the expense of Fe, has been observed to be one of the causes of this physiological deficiency (Wallace et al., 1992, Pich et al., 1994). In other cases, the complexation of Fe into insoluble chelates by heavy metals, coupled with the often acidic conditions of the contaminated soil, has been reported to be the cause of iron deficiency (Wallace et al., 1992, Ma et al., 2011a).

*(iii) Imbalances in plant hormone secretion*

Heavy metal tolerant plants that are being used for phytoremediation may suffer hormonal imbalances due to the toxicity and acidic nature of the contaminated environment (Moya et al., 1995, Kanwar et al., 2012). Ethylene is a plant hormone of which a minimum amount is required to promote seed germination and root elongation (Ping and Boland, 2004) amongst other properties. Heavy metal toxicity, acidity and water stress, that is also typical of contaminated sites, may stimulate the plants to secrete ethylene in excess of the optimum amount which leads to poor root growth and overall inhibition of the plant (Mayak et al., 2004a, Maksymiec, 2007, Siddikee et al., 2011) .

### 2.6.3. Bacteria assisted phytoremediation

Plant-growth promoting bacteria (PGPB) are growth enhancing symbiotic bacteria that live on or around the plant root system (rhizosphere) and significantly promote

overall plant growth while deriving nutrients and other benefits from the plant (Vessey, 2003, de-Bashan et al., 2012).

PGPB are broadly divided into two types: endophytes and rhizobacteria. Endophytes are bacteria that aggressively colonise (through a plant permissible symbiotic mechanism) and penetrate the plant roots to form nodules, whereas rhizobacteria are symbiotic bacteria that migrate from the bulk soil to live in the rhizosphere but do not necessarily penetrate the plant root (do Vale Barreto Figueiredo et al., 2010, Compant et al., 2010). Apart from promoting the growth of plants, some PGPB have been found (as a free living microorganism) to function as a microbial-bioremediator by influencing the bioavailability of heavy metals through the release of chelates and moderation of soil pH and Eh (Lucy et al., 2004, Smith and Read, 2008). The combination of PGPB with hyperaccumulators/phytostabilisers has been recently recognized as a more efficient system in the sequestration of toxic heavy metals (Ma et al., 2011a, Marques et al., 2013, Qiu et al., 2014) and is the focus of my PhD research.

### 2.7. Roles of PGPB

Research to identify the role or roles that PGPB perform in plant-microbe symbiotic relationships in contaminated environments is ongoing worldwide. Despite inconclusive and diverse results, possible roles of PGPB in metal phytoremediation are summarised in Figure 2.5 and discussed as follows.

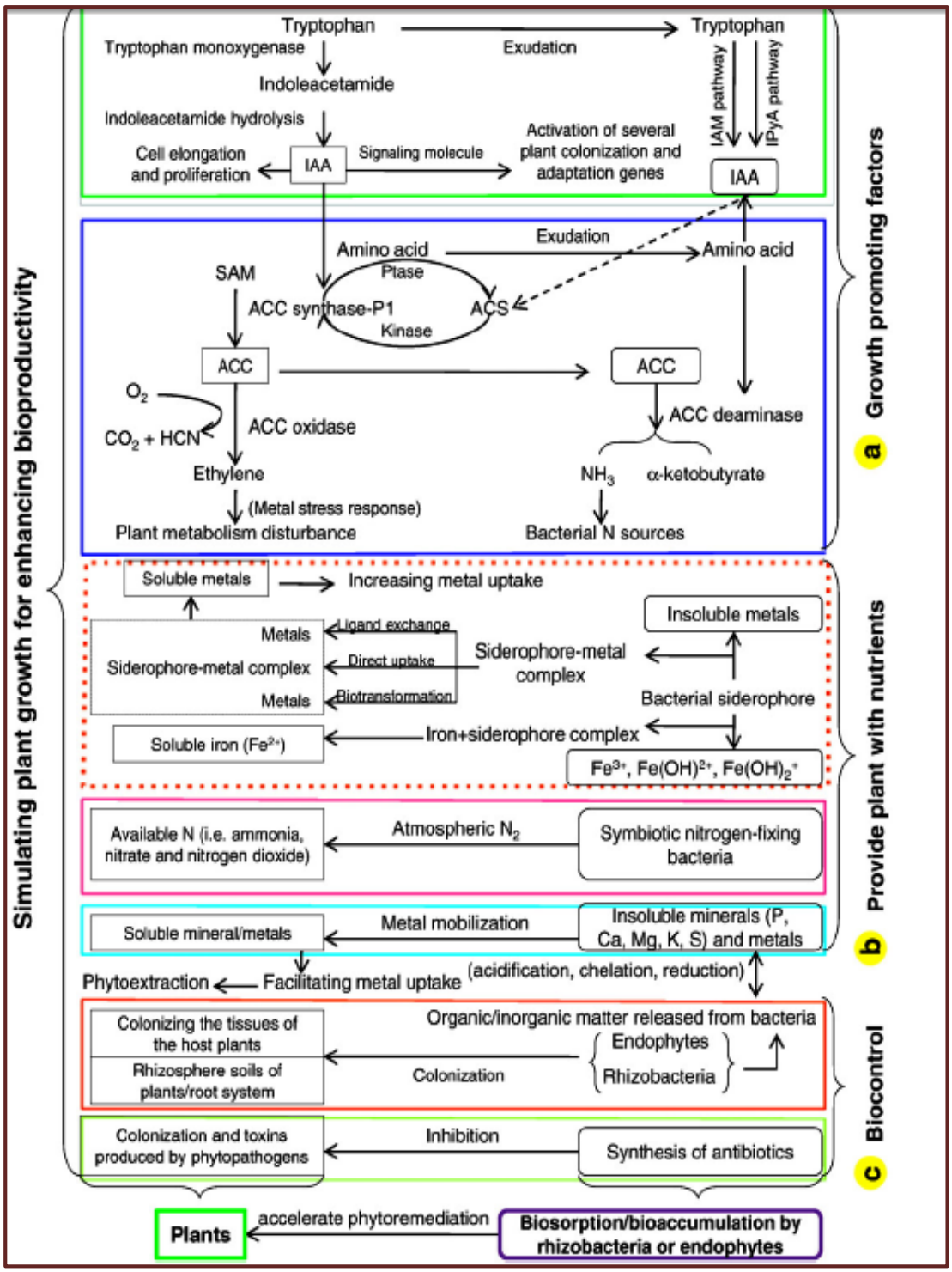



**Figure 2.5: "Plant growth promoting rhizobacteria and endophytes accelerates phytoremediation of metalliferous soils through modulation of (a) plant growth promoting parameters, (b) by providing plants with nutrients, and (c) controlling disease through the production of antifungal metabolites. Abbreviations: indole-3-acetic acid (IAA), indole-3-acetamide (IAM) pathway, indole-3-pyruvate (IPyA) pathway, methionine-S-adenosylmethionine (SAM), 1-aminocyclopropane-1-carboxylate (ACC), 1-aminocyclopropane-1-carboxylate synthase (ACS), phosphatase (Ptase), ammonia (NH3), hydrogen cyanide (HCN)." (Ma et al., 2011a)**

### 2.7.1. Amelioration of macronutrient and micronutrient deficiencies

*(i) Nitrogen fixation*

Some PGPB are capable of fixing nitrogen in soils from the atmosphere (Malik et al., 1997, Hardoim et al., 2008). Both endophytes and rhizobacteria are capable of increasing the nitrogen content of contaminated soil thus increasing the amount of the macronutrient available for plant uptake and possibly preventing or correcting nitrogen deficiency symptoms in phytoremediators (Dobbelaere et al., 2003, Wu et al., 2006, Zhuang et al., 2007).

*(ii) Solubilisation of fixed phosphorous*

PGPB have been discovered to have possibly dual positive effects in the correction of plant phosphorous deficiencies in heavy metal contaminated environments. PGPB are capable of directly releasing or solubilising the unavailable strong fixed phosphates through inorganic acidification, release of organic acids, chelation, and ion exchange reactions (Chung et al., 2005, Rodriguez et al., 2006, Saharan and Nehra, 2011). Furthermore, PGPB may enhance the mineralisation of organic phosphorous to correct phosphorous deficiency and thus promote plant growth in metal stressed environments (Zhuang et al., 2007, Van Der Heijden et al., 2008, Richardson and Simpson, 2011).

*(iii) Release of iron*

PGPB are capable of preventing the plant essential Fe from reacting with other heavy metals in the contaminated environment. PGPB produce abundant microbial siderophores that complex iron and prevent it from reacting to form insoluble hydroxides and oxyhydroxides in metal contaminated soils (Rajkumar et al., 2009, Ma et al., 2011a, Kumar and Patra, 2013). The siderophore-chelated-Fe can be

utilised by plants and therefore serves as a source of Fe for plant uptake (Wang et al., 1993, Rajkumar et al., 2010).

### 2.7.2. Maintenance of optimum ethylene level and secretion of other phytohormones

The metabolic sequence in the production of excessive ethylene ($C_2H_4$) in plants growing under environmental stress has been found to be secretion of methionine-S-adenosylmethionine (SAM), production of 1-aminocyclopropane-1-carboxylic acid (ACC), and then the production of ethylene ($C_2H_4$) (Adams and Yang, 1979, Opiyo and Ying, 2010). PGPB have been discovered to act upon ACC, the precursor of ethylene, through utilization of the nitrogen liberated from the biochemical transformation of ACC (by the bacterial enzyme *ACC-deaminase*) as their only source of metabolic nitrogen (Penrose and Glick, 2001, Rajkumar and Freitas, 2008). The transformation of ACC therefore reduces the amount of ethylene produced and thus moderates the inhibitory effects of ethylene on plants growing in heavy metal contaminated soils (Glick et al., 1998, Belimov et al., 2002). The moderation of ethylene levels in plants by PGPB ensures optimum root development, a desirable plant growth characteristic in phytostabilization (Grandlic et al., 2008, Rajkumar et al., 2012). PGPB have also been found to enhance the plant's ability to produce other growth essential hormones, such as cytokinins and gibberellins which ensure optimum seed germination and meristemic development in heavy metal contaminated environments (Li et al., 2007, Taghavi et al., 2009, Ma et al., 2011a).

### 2.7.3. Direct promotion of plant metal uptake or alleviation of metal toxicity by PGPB

Apart from conferring resistance and ensuring optimum plant growth, PGPB have been found to indirectly and directly promote plant uptake of heavy metals. Through the production of siderophores (Braud et al., 2009), bisurfactants (Braud et al., 2006) and organic acids (Saravanan et al., 2007), it has been suggested that PGPB promote the bioavailability of heavy metals and enhance their uptake by the plant. The enhanced uptake reduces phyto-toxicity of metals to roots through increased root to shoot metal translocation reducing critical metal concentration in the root and subsequently promoting plant growth (Rajkumar et al., 2006, Wu et al., 2006).

Moreover, the direct microbial biosorption (negatively charged surface area) and bioaccumulation into the cells of highly abundant bacteria (Ledin et al., 1996) of heavy metals, decreasing the uptake of toxic heavy metals by plants, has been cited as a key mechanism for significant plant growth promotion that often occurs in a microbial-phytoremediation system (Heggo et al., 1990, Jing et al., 2007). Examples of plant growth promoting bacteria, the phytoremediating plants they promoted and the possible plant growth promoting mechanism is presented in Table 2.4.

**Table 2.4: Examples of Plant Growth Promoting Rhizobacteria (Zhuang et al., 2007)**

| Bacteria | Plant | Heavy metal | Condition | Role of PGPR | Reference |
|---|---|---|---|---|---|
| *Azotobacter chroococcum* HKN-5<br>*Bacillus megaterium* HKP-1<br>*Bacillus mucilaginosus* HKK-1 | *Brassica juncea* | Lead, zinc | Pot experiments in greenhouse | - Stimulated plant growth<br>- Protected plant from metal toxicity | Wu et al. (2006b) |
| *Bacillus subtilis* SJ-101 | *Brassica juncea* | Nickel | Pot experiments in growth chamber | - Facilitated Ni accumulation | Zaidi et al. (2006) |
| *Brevundimonas* sp. KR013<br>*Pseudomonas fluorescens* CR3<br>*Pseudomonas* sp. KR017<br>*Rhizobium leguminosarum bv. trifolii NZP561* | None | Cadmium | Culture media | - Sequestered Cd directly from solution | Robinson et al. (2001) |
| *Kluyvera ascorbata* SUD165 | Indian mustard | Nickel, lead, zinc | Pot experiments in growth chamber | - Both strains decreased some plant growth inhibition by heavy metals<br>- No increase of metal uptake with either strain over noninoculated plants | Burd et al. (2000) |
| *Kluyvera ascorbata* SUD165/26 | Canola<br>Tomato | | | | |
| *Mesorhizobium huakuii* subsp. *rengei* B3 | *Astragalus sinicus* | Cadmium | Hydroponics | - Expression of $PCS_{At}$ gene increased ability of cells to bind $Cd^{2+}$ approximately 9- to 19-fold | Sriprang et al. (2003) |

## 2.8. Chapter Summary

The Chapter appraised the significance of heavy metals as important contaminants and described their toxicity and behaviour in the environment. It emphasised metal speciation as an important parameter to be considered in recommending permissible limits for heavy metals and in developing remediation techniques. Bioremediation of metal contaminated environments was identified as sustainable technology but with reduced remediation efficiency in comparison to chemical and mechanical remediation methods. The use of bacteria to promote growth of remediating plants was identified as a promising method for enhancing metal bioremediation but how bacteria simultaneously promote toxic metal bioaccumulation and growth in plants remains elusive. The understanding of the specific role of PGPB within a metal-bacteria-plant system is however crucial to the development of a timely, economical and sustainable bioremediation technology. The knowledge gained from this research can then be adopted to enhance the remediation potentials of plants used for remediating metals contaminated soils.

CHAPTER 3

# 3. Materials and methods

## 3.1. Introduction

There are over 20 toxic metal contaminants, each with different biochemical properties and health/environmental significance (Nieboer and Richardson, 1980, Fowler et al., 2011). The list of plants identified as capable of remediating toxic metals is also long. Most of the identified plants are either capable of remediating a specific type(s) of metal contaminant or exhibit different metal tolerance levels to different types of metal contaminants and they differ in anatomical and physiological characteristics (Vara Prasad and de Oliveira Freitas, 2003, Sinha et al., 2013).

Moreover, hundreds of plant growth promoting bacteria have been identified that differ with respect to growth promoting ability, metal tolerance and compatibility with plants (Glick, 2010). The soil is the natural habitat for most plants and bacteria, it is also a sink for large amounts of toxic metal contaminants (Alkorta et al., 2004). The soil system therefore exhibits enormous variability (Stenberg et al., 1998) that may make the study of the effect of a specific bacteria species on a particular metal contaminant and metal phytoremediator very difficult if not impossible. Furthermore, in the absence of a specific analytical procedure designed to comprehensively study metal-microbe processes in a dynamic soil-bacteria-plant system the need to integrate the use of analytical tools designed for geological studies, chemistry, physics, biology and microbiology was inevitable. Some of these tools are sensitive to contamination from the experimental samples, mostly requiring samples to be free of dirt and adhering soil particles.

In the light of these challenges, this Chapter presents the rationale behind the choice of research materials and methods used for the study of changes in metal speciation, bioaccumulation and enhanced soil metal remediation in plants under the influence of plant growth promoting bacteria. The Chapter also provides details of methods that are referred back to in later Chapters. Results from the study are presented in Chapter 4, 5, 6 and 7 where some of these methods will also be outlined.

## 3.2. Research materials

### 3.2.1. Choice of plants

*(i) Brassica juncea (L.) Czern (*common name: *Indian mustard)*

Of all plants identified for metal remediation, plants from the family *Brassicacea* clearly stand out for their inherent metal tolerance and remediation abilities, and are arguably the most prominent when it comes to practical use in contaminated site remediation (Palmer et al., 2001, Anjum et al., 2012, Mithen, 2013). *Brassica juncea (L.) Czern*, a member of the *Brassicacea* family is a well-researched metal accumulator used for the remediation of a variety of contaminants like Cr (Rajkumar et al., 2006), Zn (Ebbs et al., 1997), Cu (Ebbs and Kochian, 1997) and Pb (Begonia et al., 1998). Moreover, the roots of *Brassica spp*. naturally harbor several beneficial bacteria and can also easily be colonised with inoculated plant growth promoting bacteria species (Belimov et al., 2005, Ahmed et al., 2012). *B. juncea* (Figure 3.1) is an annual vascular plant and completes its life cycle within 2-3 months.

**Figure 3.1: *Brassica juncea* plant**

These characteristics therefore make *B. juncea* ideal for this PhD research to ensure repeated growth experiments are conducted and evaluated within the specified period of three years. Seeds of *Brassica juncea* plants were purchased from Sow Seeds Ltd. Cheshire, UK, a seed merchant certified by Defra UK.

*(ii)* *Vicia sativa* (common name: cultivated vetch)

*Vicia sativa* subsp. *sativa* L. also known as cultivated or garden vetch is a leguminous plant from the plant family *Fabaceae* (Zohary and Plitmann, 1979, Samarah et al., 2004). It is an annual scrambling and climbing plant with a thin stem (Figure 3.2) and slender but highly branched tap roots (Sattell et al., 1998).

**Figure 3.2*: Vicia sativa* plant**

Although its metal tolerance or remediating ability is relatively un-known, it excels in its ability to host diverse population of bacteria species (Lei et al., 2008, Pasca et al., 2012). Like *B. juncea, V. sativa* also completes its life cycle within 2-3 months. It was also sourced from Sow Seeds Ltd. Cheshire, UK, a seed merchant certified by Defra UK.

### 3.2.2. Choice of bacteria

*i.* *Pseudomonas brassicacearum* subsp. *brassicacearum (strain DBK11)*

Ensuring root colonisation and plant growth promotion were the main criteria that guided the choice of bacteria species for this research. Inoculating plants with bacteria isolated from the same plant species was perceived to be more likely to lead

to better bacteria colonisation and promotion of the plant's growth (Sheng et al., 2008, Ma et al., 2011a). *Pseudomonas brassicacearum subsp. brassicacearum* is an endophytic bacterium isolated from a Brassica plant (Achouak et al., 2000). Although little was known about the metal tolerance ability of this particular strain before the commencement of this research, other strains of *Pseudomonas brassicacearum* have been noted for their plant growth promoting ability both in contaminated and un-contaminated environments (Saleem et al., 2007, Krujatz et al., 2012).

A lyophilisate of *P. brassicacearum* was obtained from Leibniz Institute - German Collection of Microorganisms and Cell Cultures (DSMZ) with DSM number 13227. The strain is classified under Risk Group 1 of the German biosafety and biosecurity (TRBA) regulation which means the strain is safe to use in the laboratory and for field research.

*ii. Rhizobium leguminosarum bv. trifolii (strain WSM1325)*

*Rhizobium leguminosarum bv. trifolii* is a nitrogen fixing rhizospheric bacteria associated with diverse range of annual *Trifolium* (clover) species (Reeve et al., 2010). It is an aerobic, motile, Gram-negative, rod shape bacteria. *R. leguminosarum* bv. *trifolii* is renowned for its plant growth promoting ability in un-contaminated environment and it is amongst the most exploited species of root-nodule bacteria in world agriculture (Reeve et al., 2010). Although the metal resistance characteristics of strain WSM1325 was unknown at the commencement of the research, some strains of *R. leguminosarum bv. trifolii* (like strain TA1 & NZP561) have been demonstrated to exhibit a good level of metal tolerance and to maintain their plant

growth promoting activities under metal contamination (Purchase et al., 1997, Robinson et al., 2001).

*R. leguminosarum bv. trifolii* (strain WSM1325) isolated from the nodules of a clover plant was kindly provided by Dr Michael Dye of the School of Biological Sciences, University of Edinburgh, UK.

### 3.2.3. Choice of metal contaminant

Zinc is the 24$^{th}$ most abundant element on the earth crust (Zhao et al., 2012). It is a transition metal with an atomic number of 30 (Barak and Helmke, 1993). Zn has five stable isotopes ($^{64}$Zn (48.63 %), $^{66}$Zn (27.90 %), $^{67}$Zn (4.90 %), $^{68}$Zn (18.75 %), and $^{70}$Zn (0.62 %)) and exists in two oxidation states +1 or +2 in the environment (Broadley et al., 2007). Zinc exists as $Zn^{2+}$ in solution and it is redox stable under physiological conditions as a result of a complete *d*-shell of electrons (Broadley et al., 2007, Zhao et al., 2012). Furthermore due to the nature of $Zn^{2+}$ as a Lewis acid, it readily forms strong covalent bonds with sulphur (S), nitrogen (N) and oxygen (O) donors. Zinc exists in various forms of soluble salts like halides, sulphates, nitrates, formates, acetates, thiocyanates, perchlorates, fluosilicates, cyanides, alkali metal zincates and Zn-ammonia salts; as well as in soluble compounds, like Zn ammonium phosphate, Zn hydroxide and Zn carbonate (Barak and Helmke, 1993, Broadley et al., 2007). It also exists in a range of soluble and insoluble organic complexes (Broadley et al., 2007).

Apart from its important physicochemical properties, zinc is the second most abundant transition metal in organisms after iron (Fe), and the only metal represented in all six (oxidoreductases, transferases, hydrolases, lyases, isomerases, ligases)

classes of enzymes in living organisms (Broadley et al., 2007). Zinc is an essential trace element for terrestrial life, needed for optimum growth and development in microorganisms, plants animals and humans (Barak and Helmke, 1993, Broadley et al., 2007). It ensures normal performance of about 20 physiological functions like protein synthesis, DNA synthesis, immune function and cell division in organisms (Yin et al., 2012).

Although Zn is essential to life, it is highly toxic at a tissue concentration that is slightly higher than optimum in biological organisms (Chaney, 1993, Broadley et al., 2007). For example, high Zn intake in humans can cause acute adverse health symptoms like nausea, vomiting, loss of appetite, abdominal cramps, diarrhoea, and headaches and long-term exposure can result to chronic Zn poisoning leading to decreased blood copper concentration, anaemia, leukocyte rare disease, immunity damaged, weight loss, and other symptoms (Zhao et al., 2012). In metal contaminated soils and especially under acidic condition, Zn phytotoxicity is considered as the most extensive microelement phytotoxicity (Chaney, 1993). Plant leaf accumulation of $< 100$ mg Zn $kg^{-1}$ DW has been observed to hinder normal growth in some crops although the majority of susceptible plants normally exhibit visible toxicity symptoms of leaf chlorosis and necrosis at $[Zn]_{leaf} > 300$ mg Zn $kg^{-1}$ leaf DW(Chaney, 1993, Broadley et al., 2007).

Anthropogenic activities like mining, smelting and agricultural use of sewage sludge are the main sources Zn contamination in the environment (Romeo et al., 2014). It was estimated that approximately 10 000 tonnes Zn $yr^{-1}$ were emitted worldwide

as a result of mining and smelting activities about two thousand years ago (Nriagu, 1996). Results of an inventory of heavy metal inputs to agricultural soils in England and wales showed that zinc was the metal deposited on soil in the largest amounts from the atmosphere (Nicholson et al., 2003). The agricultural use of sewage sludge and manure is a well-established and regulated practice in the UK. Nevertheless, it was revealed that approximately 1,900 t of Zn were applied in livestock manures to agricultural land in England and Wales in 2000 (Nicholson et al., 2003).

Moreover, due to its geochemical mobility in surface water, Zn was recently identified as the most commonly encountered pollutant metal in the surface waters of metal mining regions in the UK (Gozzard et al., 2011). In a survey of top soils in 26 European countries, it was revealed that among all metals of health and environmental concern, zinc concentration was the highest (Lado et al., 2008).

Zinc was therefore chosen as the toxic metal contaminant for this research because of its complex speciation, its global environmental significance, unique physicochemical properties and its biochemical importance in plants. Zn predominantly exists as Zn sulphide and Zn oxides in the environment (Zhao et al., 2012). Previous studies of Zn speciation in plants used Zn sulphate solution as the source of Zn contamination, Zn sulphate solution was therefore used as the source of Zn contamination for this research (Sarret et al., 2002, Kopittke et al., 2011). Although sulphur is an essential secondary macronutrient for *B. juncea* plant, the possible plant growth promoting effects of 148.0 – 98.5 mg $kg^{-1}$ sulphur addition through $ZnSO_4$ application in this research would be the same across the Zn contaminated treatments.

### 3.2.4. Growing media

*i. Scotts Levington F2+S Seed & Modular growth medium*

In order to adequately quantify and understand the specific effects of selected bacteria and plant species on the chosen metal contaminant, the enormous physicochemical variability that is normally associated with contaminated soils on the field needs to be reduced and the growth medium needs to be homogeneous across all experimental treatments. Scotts Levington F2+S Seed & Modular growth medium was used in glasshouse pot experiments. It is a standard soil system for most pot experiments (Vicente et al., 2012, Llewellyn et al., 2012). It was purchased from Green-tech Ltd, UK. From the information provided by the manufacturer, it has particle size diameter of <3 mm, bulk density of 0.1 g $cm^{-3}$, pH 5.5-5.7, conductivity 210-290 µS $cm^{-1}$ and nitrogen (N), phosphorus (P) and potassium (K) content of 350, 650 and 550 mg $kg^{-1}$ respectively. The pH (in water) and NPK contents of the medium were further confirmed through laboratory analysis to be 5.8 ±0.1, 355±10 mg $kg^{-1}$, 645±15 mg $kg^{-1}$ and 555±10 mg $kg^{-1}$ (mean ± standard error of n=3 analyses) respectively before use. N and P concentrations were determined by standard automated colorimetric method and K was determined using flame atomic absorption spectroscopy (Carter, 1993). The choice of fertile well homogenised soil is to ensure the plant does not suffer from anything else apart from stress induced by metal toxicity. The background soil Zn content established through laboratory analysis was 48±10 mg $kg^{-1}$ (mean ± standard error of n=3 analyses) a concentration optimum for plant growth (Vicente et al., 2012, Llewellyn et al., 2012). The soil was sterilised and dried in an autoclave to ensure that the inoculum was not out-competed and experimental pots are of same moisture contents at the start of the experiments.

*ii. Tork advanced wiper 420 centerfeed roll M2 System*

Although the soil used in the glass house experiment was sterilised, the plants were grown in a non-sterile glasshouse environment. Some airborne bacteria species has been observed to be metal tolerant and capable of influencing metal chemistry (Ahmed et al., 2000, Fasim et al., 2002). The effects of the selected bacteria strains on plants established on a sterile growth medium was therefore further investigated under aseptic experimental conditions.

Sterile Tork advanced wiper 420 centerfeed roll M2 System was placed in sterile petri dishes and used as a medium for germinating and growing seedlings for up to 14 days under completely sterile conditions (Figure 3.3).

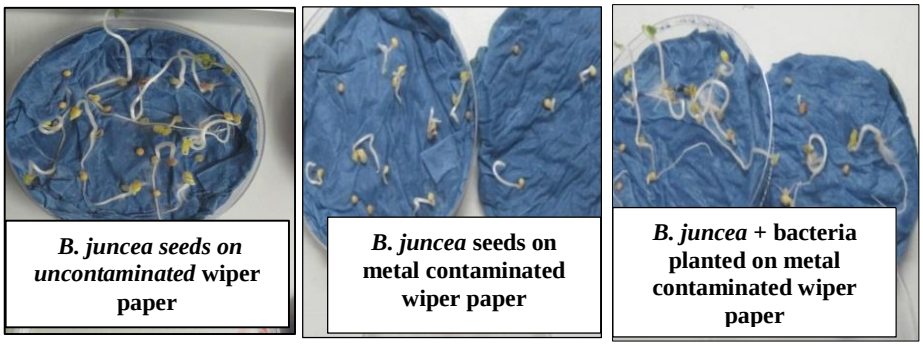



**Figure 3.3: Photographic illustration of seed germination and seedling growth on wiper paper, 10 days after planting. Figure shows effect of metal contamination and bacteria inoculation on seed germination and seedling growth an indication that the wiper is good enough to be used as a growth medium for this experiment**

Sterile Tork advanced wiper was chosen as a growth medium because it was readily available, cheap, easy to disposed and recycled. The medium was able to sustain *B. juncea* plant growth for 14 days after seed planting without the need of nutrient

supplements. The media was kept moistened through the experimental periods with sterile deionised water.

This seed germination and seedling growth method is similar to the roll towel test (Ma et al., 2011b) and the plant growth promotion assay on filter paper developed by (Glick et al., 1995) and modified by (Belimov et al., 2001). The method has been used to access plant root growth promotion activities of bacteria in inoculated *B. juncea* plants exposed to cadmium toxicity (Belimov et al., 2005) and in *Orychophragmus violaceus plants exposed to Zn* (He et al., 2010).

### 3.3. Bacteria culturing

All bacterial handling (culturing and seed inoculation) were conducted under a sterile environment in BioAir microbiological safety cabinet (Aura B4 model). All materials used for microbial analysis were sterilised for 15 minutes at 120$^{o}$C and 15 psi pressure. Sterilised materials were dried under UV light in the BioAir safety cabinet.

Glycerol stock: To ensure long term storage of bacteria culture glycerol stocks of *P. brassicacearum and R* leguminosarum were made. 50% glycerol solution in distilled water was autoclaved and allowed to cool under BioAir microbiological safety cabinet. About 300 µl of the sterile glycerol was pipetted into 2 ml cryo tubes placed on a block of ice. A sterile inoculation loop was used to harvest biomass of the bacteria into the glycerol solution in the appropriate amount (to an end bacteria concentration of 10-15% of the suspension). The mixture was gently stirred with sterile inoculation loop as it cools on ice. About 10 glycerol stocks of each bacterium were prepared. The stocks were kept frozen at -80$^{o}$C.

Plate culture: This research uses bacteria grown from a representative colony of the bacteria strain. Plate culturing is the essential procedure for obtaining single bacteria colony and the basis for evaluating biochemical responses in individual bacteria cells (Lederberg and Lederberg, 1952). Nutrient media for petri dishes was made by dissolving 28 g $l^{-1}$ Nutrient Agar (Fluka) in deionised water. The Nutrient agar was completely dissolved in deionised water and sterilised by autoclaving. Sterilised agar were poured into petri dishes and allowed to solidify under the BioAir microbiological safety cabinet. An inoculation loop was sterilised in a gas flame and used to transfer a scoop of the bacteria from the stock to the agar. The sterilised inoculation loop was repeatedly used to make streaks on the plate as appropriate, to ensure isolated growth of distinct colonies on the plates (Wistreich, 2003). The inoculated petri dishes were sealed with paraffin and incubated at 30-40$^{o}$C for 2 days.

Liquid culture: Culturing bacteria in liquid nutrient media is essential for the growth and multiplication of isolated pure colony. Metal resistance and tolerance in bacteria is also mostly evaluated in liquid media culture (Yilmaz, 2003, Rathnayake et al., 2013). Nutrient media in 250 ml flasks are prepared by dissolving 1.3g of Nutrient Broth No. 3 in 100 ml of deionised water and autoclaving. The nutrient broth media was allowed to cool and a sterilised inoculation loop was used to transfer a bacteria colony from a plate culture into the nutrient broth. Bacteria from a previous liquid culture can also be transferred to a fresh nutrient broth by pipetting 100 µl of the previous into the new flask. Flasks with inoculated cultures were capped with foam bungs, appropriately labelled and placed on a side-to side shaker to grow at 150 rpm and 30 $^{o}$C for 2 days. All bacteria liquid cultures were grown in a dedicated culture room designed to ensure stable temperature of 30 $^{o}$C.

### 3.4. Bacteria harvesting, batching and washing

Biomass from a viable colony of bacteria cells cultured in nutrient broth at 30°C to an exponential bacteria growth stage were harvested, washed and batched before further use as inoculum for seeds/soil. To harvest bacteria biomass from liquid cultures, the cultures are centrifuged at 8000g for 30 min at 5$^{o}$C in a *Sorvall™ RC 6* Plus Centrifuge *(*Thermo Scientific). Harvested biomass from each of the centrifuge tubes were batched together and washed 3 times. The first washing process involved re-suspending and stirring (with a sterile magnetic stirrer) the batched cells in sterile deionised water for 5 min. The bacteria-sterile water suspension is then re-centrifuged, batched and then washed once 1.0 M $NaClO_4$ electrolyte and one more time in deionised water to complete the 3 washing cycles. The washing process was done to ensure harvested bacteria cells are free from metallic minerals and other nutrients that are present in the media in which they were cultured.

### 3.5. Assessment of bacteria tolerance to metal toxicity

The susceptibility of the selected bacteria species in Zn contaminated environment was relatively unknown. They were chosen purely because of their perceived plant growth promoting abilities in uncontaminated environment. Their metal tolerance ability was therefore evaluated by first growing a pure colony of each bacterium species in 100 mL of standard nutrient broth placed on a side to side shaker at 30°C for three days. This was done to first achieve optimum microbial population in the media before exposing the bacteria to the contaminant. 1 ml of the bacteria-nutrient media suspension was then added to 100 mL of nutrient media contaminated with $ZnSO_4.7H_2O$ at a Zn concentration range of 200 mg $L^{-1}$ to 1000 mg $L^{-1}$ of Zn.

Bacteria viability under Zn toxicity was monitored over the period of 7 days. Viable cell populations were evaluated in triplicates at 24 hours, 48 hours and 144 hours in the 100 ml of Zn contaminated media by appropriately plating 0.1 mL of the bacterial suspension in standard nutrient agar and incubating for two days. The periods of exposure were selected to represent the period of seed germination and seedling emergence for *B. juncea* and *V. sativa* plants which is between 3 to 5 days, in order to ensure that the bacteria would survive and colonise roots in the contaminated soil.

### 3.6. Surface sterilisation of seeds

Washing and sterilisation of seeds are important procedures to ensure seeds are clean and free of any other bacteria apart from the ones inoculated. Most effective washing and sterilisation processes, however involve the use of chemicals, some of which may be toxic to seeds embryo or cause genetic mutation. For this research, a washing and sterilisation process with non-lethal but effective concentrations of sterilising agents that were targeted at sterilizing only the surface of the seeds was used. To prevent loss of seeds, all reagents used at every stage of the washing process were pipetted away from the seed-solution suspension instead of being decanted. Seeds of *B. juncea* and *V. sativa* were first washed 3 times in sterile deionised water for 10 minutes. Seeds were then soaked in 0.05 M sodium hypochlorite ($NaOCl_4$) electrolyte for 30 min and then washed once more in sterile deionised water for 5 minutes.

Sodium hypochlorite is an effective antimicrobial agent (Chun et al., 1997). This method of seed washing and sterilisation has been widely used for plants like

Brassica spp. without any report of adverse effects to seed germination, seedling growth or to plants biological composition (Chen et al., 2004, Kumar et al., 2008, Asaduzzaman et al., 2014). Sterilised seeds were then air-dried under the sterile cabinet at 30 $^{o}$C. This was to ensured optimum imbibition of inoculants by the seeds at the bacteria inoculation stage.

### 3.7. Bacteria inoculation

Seed and soil inoculations are standard methods of plant inoculation in bacteria assisted phytoremediation (Burd et al., 2000, Kumar et al., 2008). A multi-process phytoremediation processes using more than one bacteria species often uses both methods, one for each species (Huang et al., 2005). It is important to assess the optimum microbial biomass for inoculation. Too little of bacteria concentration may have no growth promoting effect whereas too much of inoculants may hinder seedling growth. For this research, a fresh microbial biomass of approximately $7.5 \times 10^{8}$ cfu ml$^{-1}$ was found to be optimal after a series of experimental tests with different concentrations. This inoculant concentration is equivalent to a microbial dry biomass of 0.5 mg mL$^{-1}$ and it is prepared by diluting batched and washed bacterial cells in sterile-deionised water to an optical density of 0.7 measured with a M501 Single Beam Scanning UV/Visible spectrophotometer at 600 nm.

For seed inoculation, sterilised seeds were soaked in the bacteria suspension under aseptic conditions at 30°C for 3 hours. Seeds for the control treatments were soaked in sterile deionised water and placed in the same environment for the same duration. For growth media inoculation, sterilised growth media was inoculated with a bacteria suspension of 0.5 mg ml$^{-1}$ dry biomass at the rate of 0.05 ml g$^{-1}$ of growth media. There were no statistical differences in plant growth promotion between the two

inoculation methods. These methods of bacteria inoculations have been widely used in bacteria assisted phytoremediation research (Burd et al., 2000, Kumar et al., 2008).

### 3.8. Plant growth experiments in sterile conditions

In order to precisely quantify the plant growth promoting ability of the selected bacteria species in both uncontaminated and metal contaminated environment, the experimental system needs to be established under an aseptic condition. Growth experiments were therefore conducted in a laminar flow hood maintained under a temperature of 25°C with artificial light (Figure 3.4)

**Figure 3.4: Description of experimental set up for plant growth experiment in a laminar flow hood. Seeds were planted on sterile wiper placed in petri dishes. Light was provided throughout the day and switched off over night to simulate day and night photo effects**

5g of sterile wiper paper (folded as 2.5 g into the base and cover of the Petri dishes respectively) were placed into sterile petri dishes (Figure 3.5).

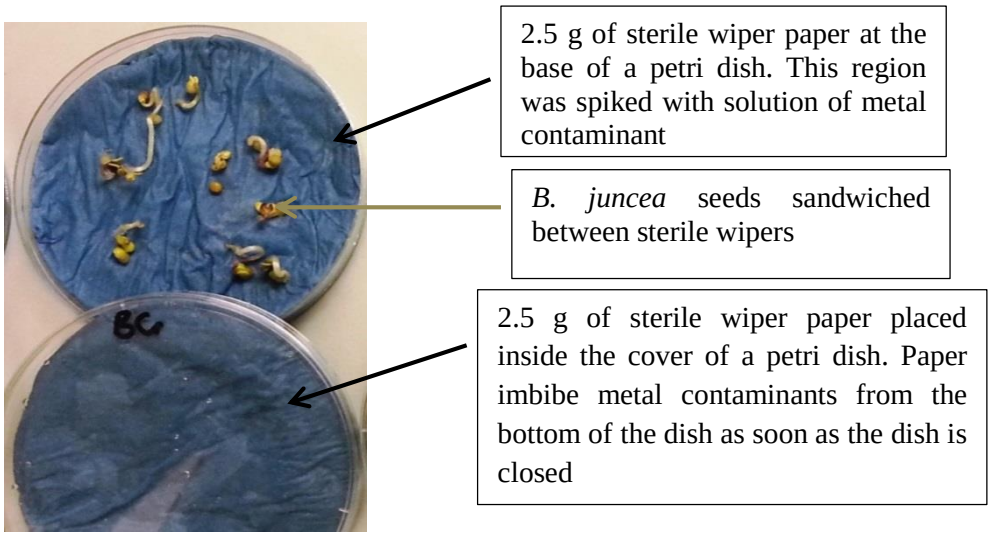



**Figure 3.5: Photographical illustration of the planting system used for evaluating plant growth promoting ability of bacteria under sterile condition**

The paper filled Petri dishes were exposed to UV light for 30 min to ensure the death of any bacteria that might have contaminated the system through the folding process. Zn sourced from $ZnSO_4.7H_2O$ salt was completely dissolved in sterile deionised water and filtered sterilised through a 0.22 mm filtration unit. The contaminant was applied at the rate of 300, 400 and 600 mg $kg^{-1}$ of dried sterile wiper paper. In order to ensure all treatments are under the same moisture condition, sterilised deionised water was added to the treatments without contamination at the same volume of contaminant solution that was added to the contaminated treatment. Treatments requiring bacteria inoculation were inoculated as described in section 3.7.

12 seeds were placed in 4 rows and 3 columns in the Petri dishes. A completely randomised design of 8 treatments (Table 3.1) and 6 replicates (a total of 72 seeds) was established for each of the experimental treatments.

**Table 3.1: Description of experimental treatments for *B. juncea* plants**

| Treatment | Description |
|---|---|
| Bo | *B. juncea*, un-inoculated, in media not contaminated |
| BPo | *B. juncea* , inoculated with *P. brassicacearum*, in media not contaminated |
| BRo | *B. juncea*, inoculated with *R. leguminosarum*, in media not contaminated |
| BRPo | *B. juncea*, inoculated with *P. brassicacearum* and *R. leguminosarum*, in media not contaminated |
| BZn | *B. juncea*, un-inoculated under metal contamination |
| BPZn | *B. juncea*, inoculated with *P. brassicacearum*, under metal contamination |
| BRZn | *B. juncea*, inoculated with *R. leguminosarum* , under Zn contamination |
| BRPZn | *B. juncea*, inoculated with *P. brassicacearum* and *R. leguminosarum*, under Zn contamination |

The experiment was established for 14 days and was kept moist with sterile deionised water applied at equal volume to all treatments. Seed germination rate, shoot and root lengths and plant dry biomass are growth parameters used for estimating plant growth promoting ability of bacteria in uncontaminated and contaminated environment (Burd et al., 2000, Cassán et al., 2009, Ahmad et al., 2014). Number of germinated seeds, fresh shoots and root length, total seedling lengths and total plant dry biomass were therefore assessed after the growth period of 14 days. Seeds with conspicuous radicle were regarded as germinated. Root and shoot lengths were measured in cm with a metre rule. Biomass was dried at 70$^{o}$C for 3 days to a constant weight and weighed in milligrams with a sensitive balance.

### 3.9. Plant growth and phytoremediation experiments in the glasshouse

After establishing plant growth promotion under aseptic experimental conditions, two plant growths and phytoremediation experiments were conducted in contaminated soil (Section 3.2.4). Pot experiments were conducted in a glasshouse at the School of Biological Sciences, University of Edinburgh at a minimum of 21°C daytime and 18°C night-time temperatures. Artificial lightening was used to provide a photoperiod of 18 hours $day^{-1}$, and photo levels of ~150 µmol $m^{-2}$ $s^{-1}$.

0.5 kg of growth media was placed into a 5 litre plastic pot located in a plastic saucer for each experimental replicate. The Zn contamination treatments involved spiking soil in the pots with Zn sulphate solution. Two glasshouse experiments were conducted. The first experiment was conducted in December 2011 to January 2012 with Zn contamination treatment spiked with Zn solution at the rate of 600 mg Zn $kg^{-1}$ soil DW. The experiment consisting of 8 treatments (see Table 3.1) was completely randomised in the glasshouse space and established in duplicate. The experiment lasted for 8 weeks. An illustration of the glasshouse experiment is presented in Figure 3.6.


**Figure 3.6: Photographic illustration of pot arrangement at planting (a) and plant growth at 3 weeks (b) and 6 weeks (c) after seed planting in the glasshouse.**

The second experiment was conducted in a similar manner but replicated 3 times and conducted for 6 weeks with Zn contamination treatments spiked at the rate of 400 mg Zn $kg^{-1}$ soil dry weight. Lower metal contamination and shorter experimental duration were chosen to ensure availability of sufficient biomass across the treatments for further analysis. The same volumes of deionised water were added to the treatments without metal contamination. The pots were then watered with deionised water to field capacity and allowed to stand in the glasshouse for 1 week for metal stabilization before seeds were planted.

Seeds were planted at the rate of 5 per pot. Seedlings were thinned out 5 days after emergence, to leave 2 plants in each pot. To prevent water stress the soil was kept moist throughout the experiment by adding deionised water to the saucer placed under the pots in order to prevent drastic washing down of the metal contaminant and as well prevent possible seedling damages that may be caused by the physical impacts of water on seedlings (Bakhsh et al., 1990). Although the experiment was designed to be a closed experimental system, there was possible loss of Zn through the watering process (i.e. accidental over-watering of the saucer placed under the perforated experimental pots), thinning of plants and weeds removal. Efforts were however made to reduce Zn loss from the experimental system as much as possible. For example, addition of water to the saucers was done carefully to reduce incidence of saucer over flow. The thinning of plants was done five days after immergence and pots were weeded as soon as the weeds emerged to mitigate possible Zn loss through plant biomass removal.

Plant height above the soil surface was measured weekly. Above ground and below ground dry biomass per pot was also determined after the experiments by harvesting

the biomass, rinsing in deionised water and drying at 70$^{o}$C to a constant weight. Soil and plant samples were analysed. These analyses are described as follow

(i) Metal bioaccumulation and phytoremediation analysis from pot experiment (II):

Zn accumulation by the plants in different treatments from the second glasshouse experiment was assessed by chemically extracting total Zn concentrations in the harvested above and below ground plant biomass through a standard wet acid digestion method (Lowther, 1980). 0.1g of dried, ground and sieved plant biomass was accurately weighed into Pyrex digestion tubes, and the biomass was digested with 2 ml of concentrated sulphuric acid and 1.5 ml of hydrogen peroxide. The mixture was gradually heated in a digestion chamber to a temperature of 30$^{o}$C until it turned colorless. The clear supernatant was transferred to a graduated flask and made up to 100 ml with deionised distilled water.

The soil in each of the 3 replicate pots per treatments was sampled at the end of the experiment and two 1 g oven-dried sub-samples were sequentially extracted into 4 fractions using a modified Community Bureau of Reference (BCR) sequential extraction scheme (Rauret et al., 2000) to determine exchangeable (readily bioavailable), reducible and oxidisable soil Zn fractions and the residual soil Zn. Acetic acid (0.11 mol $l^{-1}$), hydroxylamine hydrochloride (0.5 mol $l^{-1}$), hydrogen peroxide (8.8 ml $l^{-1}$) and ammonium acetate (1.0 mol $l^{-1}$) were used to obtain exchangeable, reducible and oxidizable Zn fractions in 1g of soil respectively

(Rauret et al., 1999). The residual Zn content was extracted in aqua regia, a 1:3 mixture of nitric and hydrochloric acids for 3 hours at 110$^{o}$C (Rauret et al., 2000).

All extracts were analyzed using inductively coupled plasma-optical emission spectroscopy (ICP-OES) using a Perkin Elmer Optima 5300 DV. Zn calibration standards prepared from analytical grade AAS standards were used to check accuracy against a certified multi-element M6 (VWR) standard among sample runs. Precision of analysis was checked by calculating percentage Relative Standard Deviation (RSD) (Eggins et al., 1997, Chen and Ma, 2001) within sample means at every analytical stage (Equation 3.1).

Precision (%) = [(standard deviation of means)/means] x 100 (3.1)

Accuracy of analysis was checked by calculating percentage bias between the concentrations of reference standards and measured concentrations (Equation 3.2) (Chen and Ma, 2001).

Accuracy (%) = (conc. of standards – measured conc.)/ (conc. of standards) (3.2)

Using these, the error on Zn determination was less than 10%. An analysis of precision and accuracy of the four steps sequential Zn determination process is presented in See Table 3.2.

Table 3.2: Analysis of precision and accuracy of the four steps sequential Zn determination process

| Analytical runs | Reference standards mg $l^{-1}$ | Mean n = 6 mg $l^{-1}$ | Standard error of mean | Standard deviation of mean | Variance of mean | % Precision | % Accuracy between reference standards and measured values |
|---|---|---|---|---|---|---|---|
| Step 1 extraction | 0.2 | 0.1938 | 0.00657 | 0.01138 | 0.00013 | 5.87 | 3.1 |
| | 0.5 | 0.4741 | 0.0131 | 0.0226 | 0.0005 | 4.77 | 5.18 |
| | 1.0 | 0.9936 | 0.0112 | 0.0194 | 0.0004 | 1.95 | 0.64 |
| | 2.0 | 1.9973 | 0.0663 | 0.1148 | 0.0132 | 5.75 | 0.135 |
| | 5.0 | 4.64 | 0.108 | 0.188 | 0.035 | 4.05 | 7.2 |
| | 10 | 10.221 | 0.0209 | 0.0362 | 0.0013 | 0.35 | 2.21 |
| Step 2 extraction | 0.2 | 0.1989 | 0.0107 | 0.0185 | 0.0003 | 9.28 | 0.55 |
| | 0.5 | 0.4845 | 0.021 | 0.0364 | 0.0013 | 7.51 | 3.1 |
| | 1.0 | 1.0028 | 0.0115 | 0.0199 | 0.0004 | 1.99 | 0.28 |
| | 2.0 | 1.988 | 0.00422 | 0.00731 | 0.00005 | 0.37 | 0.6 |
| | 5.0 | 4.8102 | 0.0715 | 0.1238 | 0.0153 | 2.57 | 3.796 |
| | 10. | 9.913 | 0.116 | 0.2 | 0.04 | 2.02 | 0.87 |
| Step 3 extraction | 0.2 | 0.19855 | 0.00605 | 0.01047 | 0.00011 | 5.27 | 0.725 |
| | 0.5 | 0.5091 | 0.0101 | 0.0175 | 0.0003 | 3.44 | 1.82 |
| | 1.0 | 1.0064 | 0.0264 | 0.0457 | 0.0021 | 4.54 | 0.64 |
| | 2.0 | 2.0586 | 0.0389 | 0.0675 | 0.0046 | 3.28 | 2.93 |
| | 5.0 | 5.097 | 0.15 | 0.259 | 0.067 | 5.09 | 1.94 |
| | 10 | 10.455 | 0.247 | 0.428 | 0.183 | 4.09 | 4.55 |
| Step 4 extraction | 0.2 | 0.19028 | 0.000592 | 0.00103 | 0.000001 | 0.54 | 4.86 |
| | 0.5 | 0.48396 | 0.00408 | 0.00707 | 0.00005 | 1.46 | 3.208 |
| | 1.0 | 0.9877 | 0.00569 | 0.00985 | 0.0001 | 1.0 | 1.23 |
| | 2.0 | 1.9415 | 0.0232 | 0.0402 | 0.0016 | 2.07 | 2.925 |
| | 5.0 | 4.9719 | 0.0521 | 0.0902 | 0.0081 | 1.81 | 0.562 |
| | 10 | 10.126 | 0.0588 | 0.102 | 0.0104 | 1.01 | 1.26 |

Extraction blanks (of the whole procedure with the extraction reagents) were also analysed and subtracted from analytical results. Means of the blank values and standard deviation of the means were calculated at every analytical stage. An average detection limit for Zn determination was calculated (by multiplying Means standard deviation (mg $l^{-1}$) by 3) (Thomsen et al., 2003) to be 0.09872 (mg $l^{-1}$) (see Table 3.3).

Table 3.3: Mean of blank values and detection limits for Zn analysis

| Experimental blanks (mg $l^{-1}$) | Mean (n=9) | Standard error of mean | Standard deviation | Detection limit (Zn mg $l^{-1}$) |
|---|---|---|---|---|
| Step 1 extraction | -0.0309 | 0.00891 | 0.02673 | 0.08019 |
| Step 2 extraction | -0.0095 | 0.0137 | 0.0412 | 0.1236 |
| Step 3 extraction | 0.0251 | 0.0102 | 0.0305 | 0.0915 |
| Step 4 extraction | 0.0275 | 0.0111 | 0.0332 | 0.0996 |
| Mean Detection limit (mg $l^{-1}$) | | | | 0.09872 |

Total Zn content was calculated as the sum of Zn concentrations in the four fractions. The sum equals the result of total Zn concentration (mean ± standard error of n=3 analyses ±10 mg $kg^{-1}$) in some selected samples extracted in aqua regia, a 1:3 mixture of nitric and hydrochloric acid for 3 hours at 110$^{o}$C (Rauret et al., 2000). Certified reference sediment and plant material were also extracted and analysed using the same analytical procedures used for the soil and plant samples. Although the nature and properties of sediments are not exactly the same with that of soils, the stream sediment reference material was the only available material that was close to the soil used for this study in the laboratory were the analysis was done. Results of the analysed reference materials were compared to their certified values (Table 3.4.).

Table 3.4: A comparison of the results of analysed reference materials with their certified values

| Reference Material | Certified values (mg $kg^{-1}$) | Analysed results | |
|---|---|---|---|
| | | Mean (mg $kg^{-1}$) | Standard deviation (mg $kg^{-1}$) |
| NCS Certified reference material DC73308 Stream sediment | 46 | 53.0 (n = 15) | 8.08 |
| IAEA-V-10 Trace elements in hay | 24 | 17.7 (n = 6) | 10.8 |

Phytoremediation efficiency was estimated by subtracting total soil Zn contents i.e. the sum of the different BCR fractions and residue fractions, rather than a measure of the total value in pots under phytoremediation from contaminated pots without plants. Soil Zn phytoextraction efficiency was estimated by calculating Zn removal percentage as: $[(C_o - C_f)/C_o] \times 100$ (3.3)

Where C*o* and $C_f$ are total soil Zn content in contaminated pots without plants and in contaminated pots under phytoremediation respectively (Bennett et al., 2003).

Zn yield in above and below ground biomass was estimated by multiplying Zn concentration in dry biomass with dry biomass weight.

The Zn bioaccumulation factor in the plants (BF) was calculated as:

$$BF = C_p/C_s \quad (3.4)$$

Where $C_p$ is Zn concentration in the total (above and below ground) harvested dry plant biomass (mg $kg^{-1}$) and $C_s$ is the total extractable Zn concentration in the contaminated soil (mg $kg^{-1}$) (i.e. the sum of the different BCR fractions and residual fractions) rather than a measure of the total value) at the end of the experiment (Zhao et al., 2003).

Zn translocation efficiency from the root to the shoot biomass of the plants was calculating as the translocation factor (TF) for each plant:

$$TF = C_{shoot}/C_{root} \quad (3.5)$$

Where $C_{shoot}$ and $C_{root}$ are the Zn concentration in the harvested plant shoots and roots (mg $kg^{-1}$), respectively (Marchiol et al., 2004).

(ii) Determination of available nutrients in soil

The soil in each of the 3 replicate pots per treatments was sampled at the end of the experiment and two 5 g fresh sub-samples were extracted for total available nitrogen (ammonium-nitrogen and nitrate-nitrogen), available phosphate, potassium, calcium and magnesium. 100 ml of 6.0% potassium chloride solution was used for extracting total available nitrogen and the other nutrients were extracted with 100 ml of 2.5% acetic acid solution (Carter, 1993). Available nitrogen and phosphate concentrations were determined by automated colorimetric method and the concentrations of potassium, calcium and magnesium were measured using flame atomic absorption spectroscopy (Carter, 1993). All analytical concentrations was corrected to the oven-dried soil weight of the samples and calculated in mg $kg^{-1}$.

### 3.10. Statistical analysis

For the experiments conducted in Petri dishes under a laminar flow hood, 12 seeds were planted in petri dishes and each of the petri dishes was replicated 6 times for each treatment (a total of 72 seeds for every treatment). Seed germination percentage, means of root/shoot length and dry plant biomass were calculated for each treatment. Means were first subjected to Anderson-Darling's normality test procedures to test for normal distribution (Razali and Wah, 2011). All treatment data sets were normally distributed (P-value > 0.05) and of equal variance. Means of growth parameter for each of the treatments were then subjected to 1-way analysis of variance (ANOVA) to identify difference within treatment means at 95% level of statistical significance.

For the plant growth results from pot experiments, statistical analysis was conducted on the mean values of the two plants per pot (n=2 in first glasshouse experiment and n=3 in the second). The means were normally distributed and significant ($p<0.05$) differences between treatments were identified by applying t-test (for means of two treatments) or 1-way Analysis of Variance (for treatment means that are more than two) followed by Tukey's multiple comparison test to means of all parameters including the results of soil and plant compositional analysis.

Means of treatments that showed significant statistical differences in all experiments were compared and ranked according to Tukey's HSD (honest significant difference) test at 95% level of significance. The adopted statistical techniques are the acceptable analytical tools in biological science and they have been widely used in evaluating plant growth under metal contamination (Ok et al., 2011, Chen et al., 2013, Yuan et al., 2014). All statistical analyses were conducted using Minitab 16 software (Minitab$^{TM}$ Inc., USA).

### 3.11. Bacteria/metal imaging in plant biomass

Colonisation of plant root by bacteria cells and the ability of the bacteria to maintain a stable relationship with the root is an important biological process for effective plant growth promotion by bacteria (Lugtenberg and Dekkers, 1999, Compant et al., 2005, Compant et al., 2010). It is therefore essential to investigate the ability of the bacteria species to colonise plant roots under metal contamination before better plant growth in inoculated plants can be attributed to the presence of the bacteria. Moreover, bacteria-metal adsorption and en-capsulation of metal by bacteria has been suggested as possible mechanisms of bacteria induced toxic metal attenuation in

plants (Rajkumar et al., 2012). But a method that can simultaneously probe bacteria distribution and metal localisation in the biomass of fresh plant exposed to metal contamination is yet to be developed.

Furthermore, it has been suggested that some specialised organelles in the cells of the plant root possess higher metal sequestration and detoxification abilities than other plant root organelles (Manara, 2012, Dalvi and Bhalerao, 2013). However, this phenomenon has not been clearly demonstrated. A method to study bacteria colonisation/localisation and metal distribution in biomass of plants exposed to toxic metals under sterile conditions and in the glasshouse was therefore adopted and a new method was also developed.

### 3.11.1. Visualisation of bacteria colonisation of plant root

Roots of plant exposed to metal contaminated soil were examined by Scanning Electron Microscope (SEM) at the end of the experiment to assess the ability of the bacteria species to colonise plant roots and survive metal toxicity till the end of the experiment. Roots collected from the middle of the pots were first subjected to a process of chemical fixation to preserve and stabilize their structure. Samples were fixed in 3.0% glutaraldehyde prepared in 0.1 M sodium cacodylate buffer at pH 7.3 for 24 hours and then washed in three 10-minute changes of 0.1 M sodium cacodylate. Samples were then post fixed in 1.0% osmium tetroxide in 0.1 M sodium cacodylate for 45 min then washed in three 10-minute changes of 0.1M sodium cacodylate buffer. Moreover, the samples need to be dehydrated to prevent shrinkage and collapse inside the high pressured sample chamber of the SEM.

This process in principle entails replacing the water in the biomass with an organic solvent. Samples were therefore dehydrated in 50%, 70%, 90% and 100% normal grade acetones for 10 minute each, then for a further two 10 minute changes in Analar acetone. Dehydrated samples were then critical point dried in a Polaron E3000 series II drying apparatus, mounted on aluminium stubs with carbon discs and coated in an Emscope SC500 sputter coater with a 10 nm thick layer of gold palladium. The samples' rhizosphere (root-soil interphase) and colonies of bacteria at the rhizosplane were visualised in a Hitachi S-4700 Scanning electron microscope.

Through the use of the SEM, images of bacteria colonisation can be acquired at high resolution and different bacteria species can be morphologically distinguished. However, the preparation process the samples must be subjected to before imaging are not ideal for studying spatial localisation of bacteria and metals in plant biomass. The process of chemical fixation, washing and dehydration would have displaced the bacteria from the spots were they are naturally localised and as well lead to a significant translocation and loss of accumulated metal contaminant. A fluorescent based optical imaging techniques that involve minimal sample disruption and preparation was therefore developed.

### 3.11.2. Bacteria/metal distribution (Fluorescent based optical imaging)

(i) Fluorescent stains

The florescent based optical imaging technique involved the use of specialised fluorescent stains of defined emission wavelength to stain a particular material (bacteria DNA or metallic ions) in the plant biomass and subsequent imaging of the stained material with appropriate light microscopes. The first task was therefore to

identify appropriate fluorescent stains to stain bacteria and metals in the plant, and to as well identify effective light microscope for imaging. The fluorescent stains used for this research are described as follows:

4', 6-diamidino-2-phenylindole (*DAPI*) fluorescent stain: DAPI is a blue fluorescent nucleic acid stain that forms a fluorescent complex when it is attached to minor grove of Adenine (A) - Thymine (T) rich sequences of DNA (Kapuscinski, 1995). The fluorescence intensity produced by DAPI stain is directly proportional to the amount of DNA present in the stained sample, with excitation/emission maximum at 358/461 nm (Coleman et al., 1981). Because of the high affinity of DAPI for bacteria DNA relative to plant DNA and its ability to fluorescent with respect to DNA mass, DAPI stains are used for counting bacteria cells and differentiating between plant and bacteria cells based on DNA content (Schloter et al., 1997, Senjarini et al., 2013). DAPI ($C_{16}H_{17}Cl_2N_5$) is soluble in water at 20 mg $ml^{-1}$, does not contains heavy metals and it is not phytotoxic (Kapuscinski, 1995). 0.8 μg $mL^{-1}$ of the stain was prepared under a sterile condition with sterile deionised water. Roots of plants exposed to toxic metal under sterile condition were sampled at the end of experiment.

Root samples were cultured in 0.8 μg $ml^{-1}$ of DAPI stain for 30 minutes at 30$^{o}$C under darkness simulated by covering the samples completely with laboratory foil. Darkness was simulated to prevent the stain from possible photo degradation.

Although DAPI was found to be effective in staining bacteria in root samples, the stain has been reported to be inadequate in differentiating between undamaged bacteria cells and damaged or dead bacteria cells (McNamara et al., 2003).

Because this research involves the use of toxic metals, it is important to adopt a more comprehensive analytical method that will be able to distinguish between the population of bacteria that survive metal toxicity from those that were susceptible and damaged by metal toxicity. The use of other sets of fluorescent stains was therefore explored.

Invitrogen Live/Dead BacLight™ Bacterial Viability kit: The kit is made up of two nucleic acid stains, green-fluorescent SYTO®9 and red-fluorescent Propidium Iodide (PI) that are combine to stain live/dead bacteria cells (Berney et al., 2007). Apart from the difference in the types of color produced when they fluoresce, the stains differ both in their spectral characteristics and in their sensitivity to cytoplasmic membrane of bacteria (Biggerstaff et al., 2006, Berney et al., 2007). The maximum Excitation/Emission spectra for SYTO9 and PI are 484/498 nm and 535/617 nm respectively (Stocks, 2004).

The green fluorescing SYTO9 is able to pass through all types of bacterial cytoplasmic membrane, damaged or undamaged and it can be used to assess total bacteria population (Leuko et al., 2004, Berney et al., 2007). However, red fluorescing PI can only penetrate and stain bacteria cells with damaged cytoplasmic membranes (Leuko et al., 2004, Berney et al., 2007). When the two stains are used in combination, the emission properties from PI will quench the fluorescent resonance energy of SYTO9 at the points where PI fluoresces, making it possible to image

bacteria with damaged cells in red while the ones without cell damages are imaged in green (Leuko et al., 2004, Berney et al., 2007).

Although debatable, bacteria with damaged cell membrane are regarded as dead bacteria (Nebe-von-Caron et al., 2000, Berney et al., 2006, Berney et al., 2007) and the Live/Dead kit has been widely used in assessing live/dead bacteria cells in many studies (Walker et al., 2004, Bais et al., 2004, Thomas and Reddy, 2013).

0.83 μg $mL^{-1}$ of SYTO9 and 5.0 μg $mL^{-1}$ of PI were prepared under sterile conditions with sterile deionised water. These concentrations were chosen based on manufacturer recommendation that PI should be used at a concentration six times that of SYTO9 (Stocks, 2004). Root samples were cultured in the stains singly and in combination for 30 minute at 30$^{o}$C under darkness simulated by covering the samples completely with laboratory foil.

RhodZin™-3, fluorescent stain for Zn: In order to achieve the objective of studying bacteria/metal relationships in inoculated plant exposed to metal contamination, another fluorescent probe that is capable of staining metallic ions and compatible with nucleic acid based stains need to be identified. Some fluorescent sensors have been manufactured, researched and recommended for measuring metal ions in living systems (Domaille et al., 2008, Hao et al., 2013, Carter et al., 2014). RhodZin-3 is an orange fluorescent indicator for $Zn^{2+}$ ions (Sabnis, 2010, Wiederschain, 2011). It is a stain that is capable of penetrating cell membranes and it has been recognized as a valuable tool for investigating the physiological consequences of $Zn^{2+}$ sequestration in living cells (Kikuchi et al., 2004, Bonanni et al., 2006).

RhodZin-3 ($C_{38}H_{38}K_2N_4O_{10}$) does not contain any toxic metals and it is soluble in water (Sabnis, 2010, Wiederschain, 2011). It has a maximum Excitation/Emission spectra of 549/576 nm (Sabnis, 2010) making it possible to combine with SYTO 9 (maximum Excitation/Emission 484/498 nm) for simultaneous imaging of Zn and bacteria in plant tissues. 5.0 μg $mL^{-1}$ of Rhodzine-3 was prepared with sterile water under aseptic conditions, and plant samples were stained and incubated in the dark for 30 minutes.

### 3.11.3. Preparation of large plant tissue for fluorescent imaging

The primary cell walls of most plants are made up of cellulose microfibrils, a diverse assortment of polysaccharides, xyloglucans, pectins, proteins and complex polyphenolic compounds (Carpita et al., 2001, Domozych, 2012). This cellular complexity is even higher in photosynthetic eukaryotes like *B. juncea* and *V. sativa* plants due to the presence of plastids which are responsible for the green pigment in the plants (Domozych, 2012, Knapp et al., 2012). Although the fluorescent stains selected for this research are capable of penetrating cells, the complexity of the cell wall and thickness of a typical root strand was observed to significantly reduce the efficacy of the fluorescent probes.

Staining and imaging a whole root strand was found to be sufficient for studying bacteria colonisation along the root strand (Figure 3.7), but not effective for probing possible bacteria penetration beyond the root sheath and most especially, organelle based metal sequestration within the plant cells.

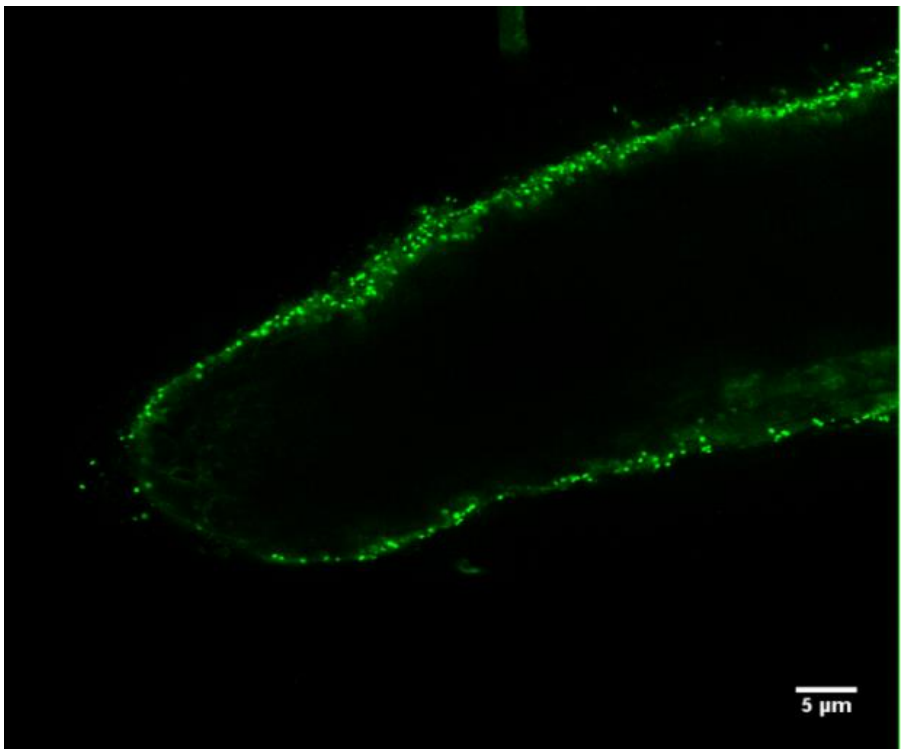



**Figure 3.7: Photographic illustration of fluorescent mapping of bacterial colonisation in whole-root of *B. juncea* exposed to zinc contamination. Figure shows bacteria around the root surface only with the complexity of the root anatomy preventing thorough study of possible bacteria penetration of the root biomass beyond the root cell wall**

Apart from the challenge of effective fluorescent staining, the use of optical microscopes to image vascular woody tissues with thick mesophyll layers and abundant chloroplasts is also problematic because any light penetrating the tissue must pass through many layers of cytoplasm, watery vacuoles, and highly refractive cell walls (Moreno et al., 2006, Knapp et al., 2012). Factors responsible for optical limitations in plants include absorbance of light, scattering of light, and severe spherical aberration resulting from refractive index changes associated with cell walls, cell contents, and air pockets (Knapp et al., 2012).

Tissue clearing has been suggested and used as strategy for deep imaging of plant tissues (Bougourd et al., 2000, Truernit et al., 2008). This method involves chemical extraction of cell wall materials and chlorophyll from the tissue, followed by

fluorescent staining of interested internal structures and infiltration of tissue with compounds that render the sample transparent to optical microscope (Truernit et al., 2008, Knapp et al., 2012). Although tissue clearing has been useful in the acquisition of high-resolution images of plant cells (Bougourd et al., 2000, Truernit et al., 2008), the protocol does not suit the objectives of this research. Most of the organelles potentially involved in metal sequestration would have been extracted away, and the possibility of washing off bacteria and metal is very high. On the other hand, sectioning plant roots to about 20 μm - 50 μm without tissue extraction has been reported to be adequate for the observation of internal cell structures (Knapp et al., 2012, Zelko et al., 2012). A cryo-histological protocol for sectioning plant root with minimum disturbance of bacteria/metal association and distribution was therefore developed and discussed as follows:

(i) Cryo-embedment of biomass in Optimum Cutting Temperature compound (OCT)

To adequately preserve the nature of the plant, the samples were first cryo-fixed in OCT before sectioning. OCT is a viscous water soluble gel composed of non-reactive ingredients, polyvinyl alcohol and polyethylene glycol (Turbett and Sellner, 1997, Weston and Hummon, 2013). Apart from assisting in tissue preservation, it makes the process of cutting easy and more comfortable to handle. A process for embedding the plant biomass under aseptic condition was first devised. Laboratory foil was sterilised in 70% ethanol for 10 min and then exposed to UV light for another 10 minutes. Cylindrical shaped foils were made by wrapping the laboratory foil around sterile glass. A strand of fresh biomass was gently placed in the cylindrical shaped foil in a horizontal position and OCT was poured in the hollow till the sample was completely covered (Figure 3.8).

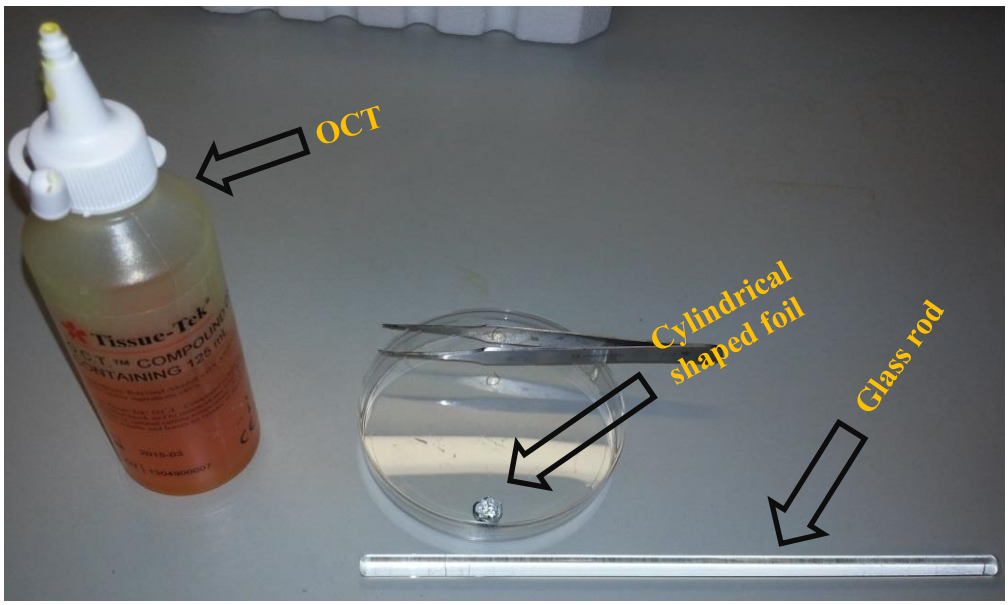



**Figure 3.8: Photographic illustration of the apparatus used for embedding root strand for cryo-sectioning**

The embedded sample was then immediately placed on liquid ice and frozen at -80$^{o}$C. Cryo-fixing the samples at -80$^{o}$C ensures immediate preservation of the natural chemistry and structure of the biomass reducing any translocation or redistribution of metal and bacteria in the plant biomass. Embedded samples can also be stored at -80$^{o}$C for up to a month without any loss of biochemical integrity (Knapp et al., 2012). OCT has been widely used in cryo-embedding biological samples for histological studies (Sun et al., 1992, Tian et al., 2011, Ludwig and Hochholdinger, 2014).

(ii) Microtome cryo-sectioning of embedded plant tissue

Cryo-embedded samples were smoothly sectioned into uniform sections with a Leica CM1900 Cryostat (Figure 3.9). The cryostat offers extremely a rapid sample freezing and accurate sectioning system for biological samples. For this research, a cryostat temperature set at -35 to -25$^{o}$C was found to be very effective for sectioning plant biomass. The Laboratory foil covering the frozen block was first removed and the

block was placed on the Cryostat's cryo-chamber. OCT was then used to mount the frozen block on the cryostat's sample holder.

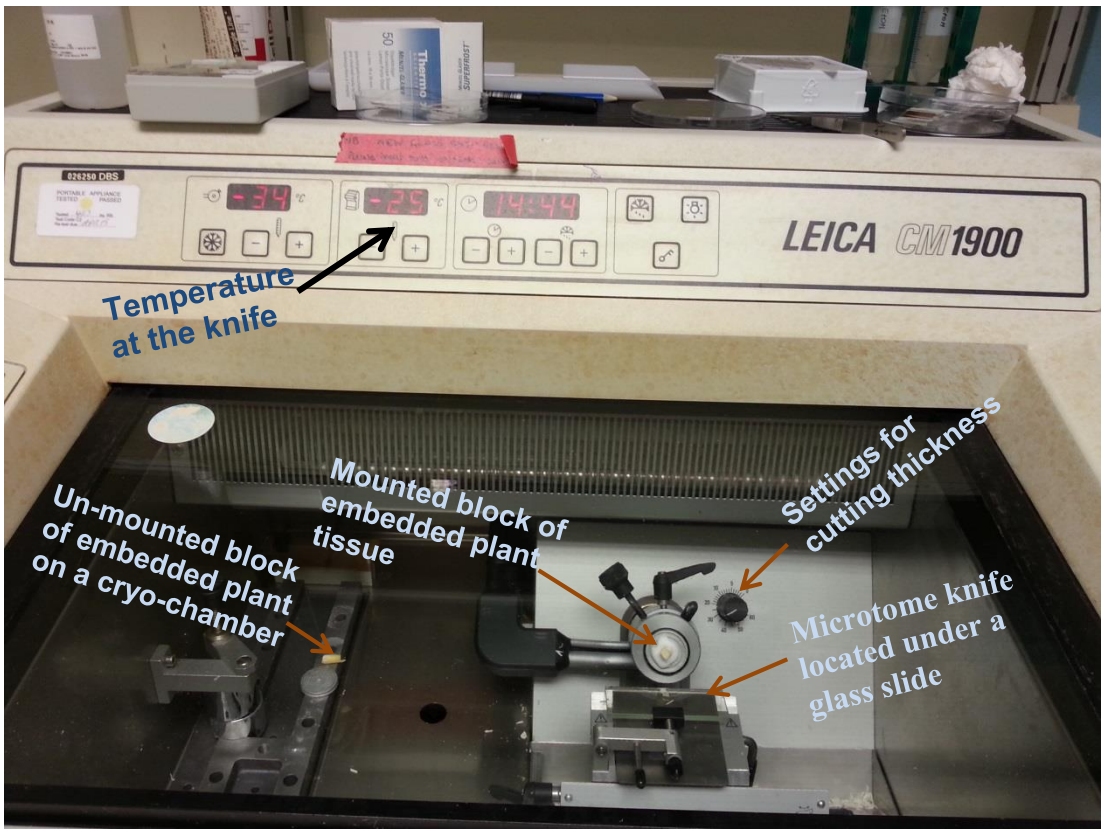



**Figure 3.9: A photographic illustration of the root sectioning process with Leica CM1900 Cryostat**

Root samples were mounted in a way that ensured sectioning is perpendicular to the root cap. Sectioning was achieved by manually rolling the Cryostat's hand-wheel which brings the mounted sample towards the knife according to the specified sectioning size. Frozen blocks that contain the sample were first trimmed at a higher size (50 µm) until the root embedded in the block was exposed. For this research, sectioned biomass was set at 35 µm thickness. Sectioned samples for microscopic imaging were collected on standard microbiology glass slides while samples to be analyzed on the synchrotron were collected on sterile Kapton tape and clipped into place with an adhesive tape.

### 3.11.4 Light and Confocal Laser Scanning Microscopy

Sectioned samples on the standard glass slides were stained with the appropriate fluorescent probe (s), covered with 20 mm x 50 mm no. 1.5 cover slips and sealed with nail polish. Prepared sample slides were imaged with two types of microscopes, a Zeiss Axio Imager and a Leica SP5 Confocal Laser Scan Microscope (CLSM) (Figure 3.10).

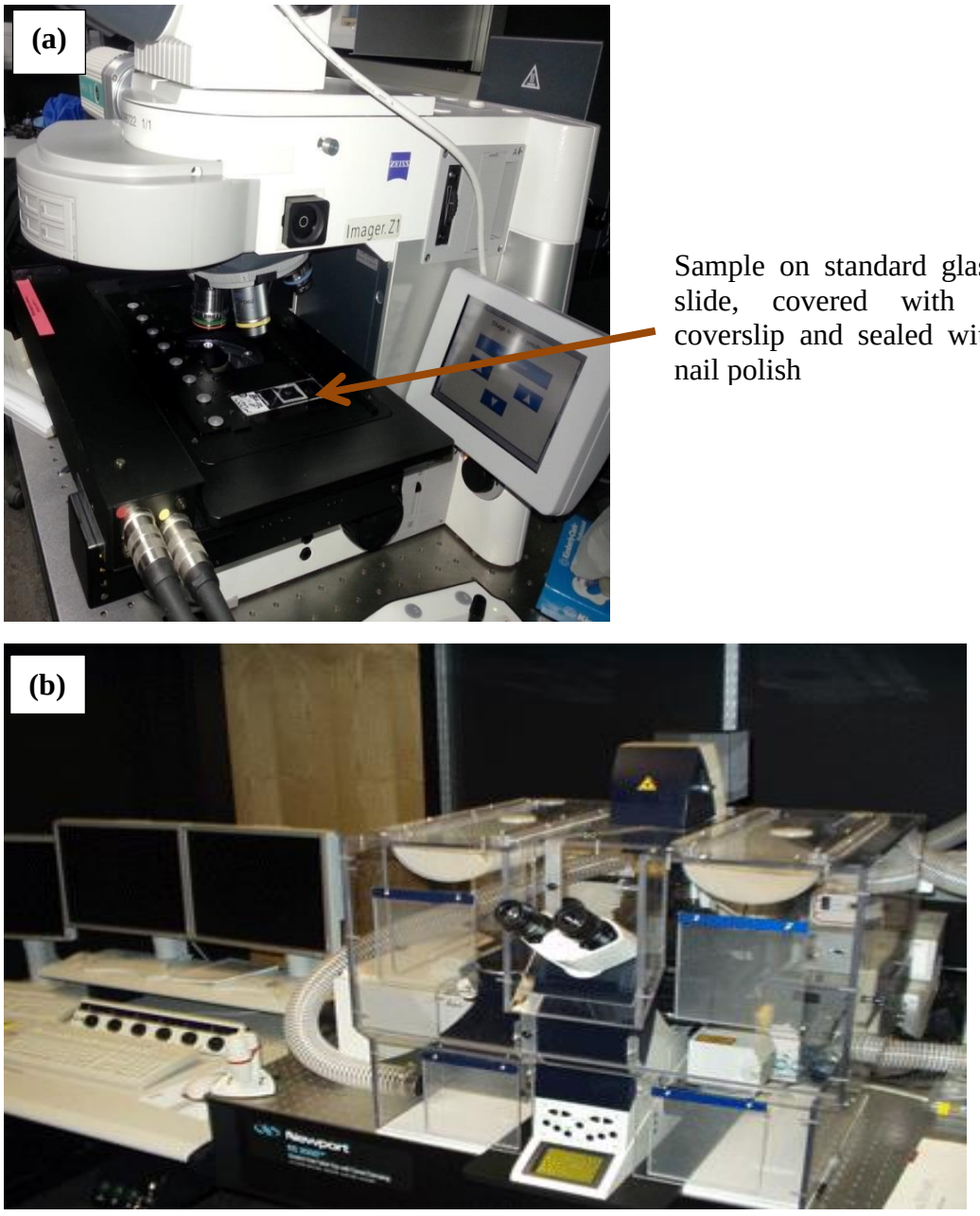



**Figure 3.10: Photographs of a sectioned sample on a glass slide and the two microscopes – (a) Zeiss Axio Imager and (b) Leica SP5 Confocal Laser Scan Microscope used in this study**

(i) Imaging with Zeiss Axio imager

Zeiss Axio imager is a Light microscope ideal for wide-field fluorescence imaging. The microscope is fitted with a digital QImaging EXi Aqua CCD camera for recording micrographs. For this research, dual band excitation filters DAPI-FITC (4′,6-diamidino-2-phenylindole and fluorescein isothiocyanate) and FITC-TRITC (fluorescein isothiocyanate and tetramethylrhodamine isothiocyanate) were used. Moreover, Zeiss 10× Plan-Achromat (Air) numerical aperture (NA) 0.45), 25× Plan NeoFluar (Oil, Water, Glycerol) NA 0.75 and x100 Plan ApoChromat (Oil) NA 1.40 were used. A graphical illustration of the quality of microscopic images with Zeiss Axio imager is presented in Figure 3.11.

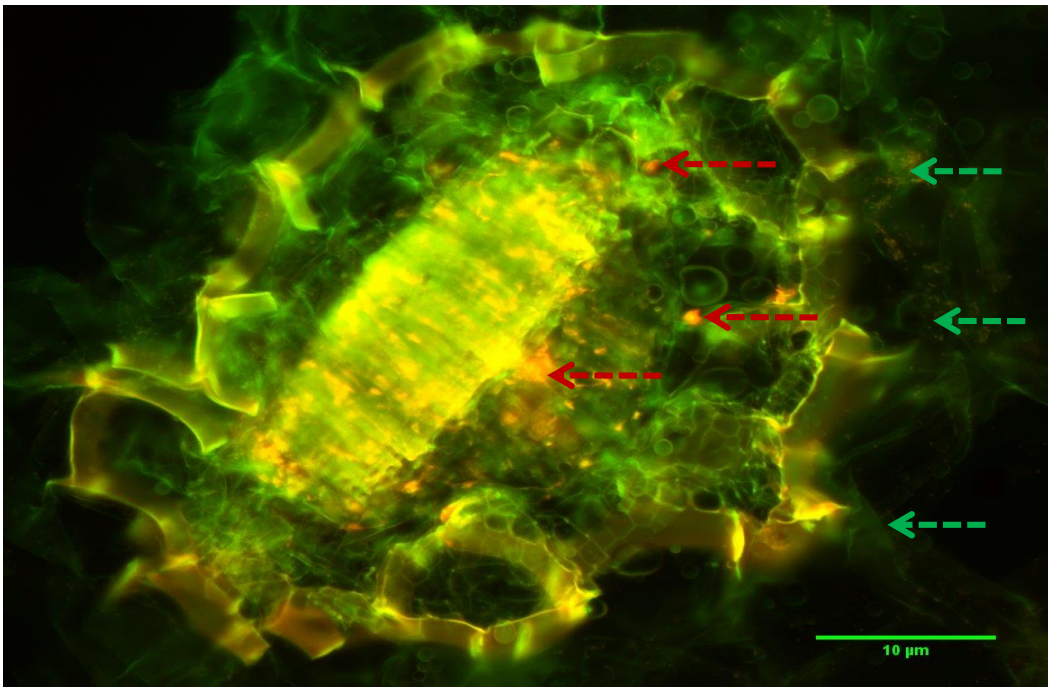



**Figure 3.11: An example of Zeiss Axio image of the cross-section of *B. juncea* root inoculated with *R. leguminosarum* and exposed to Zn contamination for 14 days. Sample was stained with Rhodzin 3 and Syto 9. Orange spots (see red arrows indicate areas of Zn localization while green spots (see green arrows) indicate bacteria cells that are not visible at this stage. Figure shows that the microscope is capable of imaging Zn distribution but its bacteria imaging ability is limited as the bacterial cells are not very visible**

The Zeiss Axio imager is indeed good enough for imaging the anatomy of sectioned plant samples and elucidating metal distribution within the sample. The resolution of the microscope is however not sufficient to clearly show bacteria structures within the sample. However, by subjecting images captured by the microscope to further processing in Image J, the silent features captured by the microscope can be enhanced and made more visible.

Image J, is a Java-based image processing program developed by the National Institutes of Health (NIH), Maryland, USA (Schneider et al., 2012). It is an open-source software focused on biological-image analysis (Collins, 2007). Bacteria features in Figure 3.11 are made more visible by subjecting the image to the 'Sharpen and Find Maxima algorithm' in Image J (see Figure 3.12).

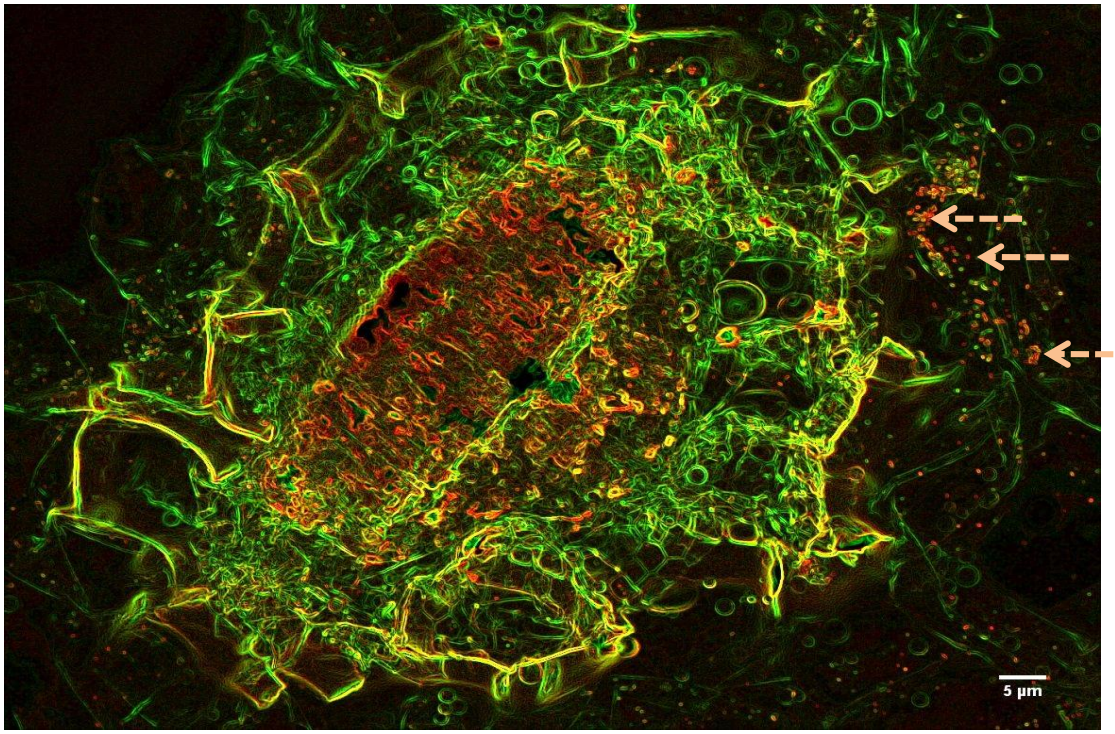



**Figure 3.12: An illustration of Image J processing (of Fig. 3.11) for magnifying bacterial cells in images acquired with Zeiss Axio imager. Image show co-localisation of bacteria (< 1.0 µm circular objects) and Zn (see arrows)**

The ‘Sharpen’ algorithm increases contrast and accentuates detail in the image while the ‘Find edges’ tool uses a Sobel edge detector to highlight sharp changes in intensity in the active image (Ferreira and Rasband, 2011). Through these enhancement procedures, bacteria cells within the root anatomy could be clearly seen.

(i) Confocal Laser Scanning Microscope

Although the Zeiss Axio imager was found useful, the Confocal Laser Scannig Microscope surpasses conventional widefield optical microscopes in terms of collecting extremely high quality images with ease. The microscope uses spatial filtering techniques to eliminate out-of-focus light or glare in samples whose thickness exceeds the immediate plane of focus (Claxton et al., 2006). Other advantages of CLSM includes reduction of image blurring from light scattering, improved signal to noise ratio, increased effective resolution and most importantly, ability to collect serial optical sections from thick sections (White et al., 1987, Paddock et al., 2014).

Access to use the Zeiss Axio imager was granted free of charge but a fee was charged for the use of the Confocal microscope. For this research therefore, the Zeiss Axio imager was used to screen and select the best samples, and the selected samples were imaged with Leica SP5 Confocal Laser Scanning Microscope to reduce the time spent on the confocal microscope and thus, the cost of research.

For all the samples imaged with the CLSM, at least 12 micrometer thick z-stacks images were acquired at a 0.5 μm z-interval. This image acquisition method affords a step-wise study of sample cross-sections along the sample’s depth. Serially collected images were then reconstructed to a 3 – dimensional image in Image J software.

An example of serial optical sections from CLSM and subsequent 3 D reconstruction is presented in Figure 3.13 and 3.14 respectively.

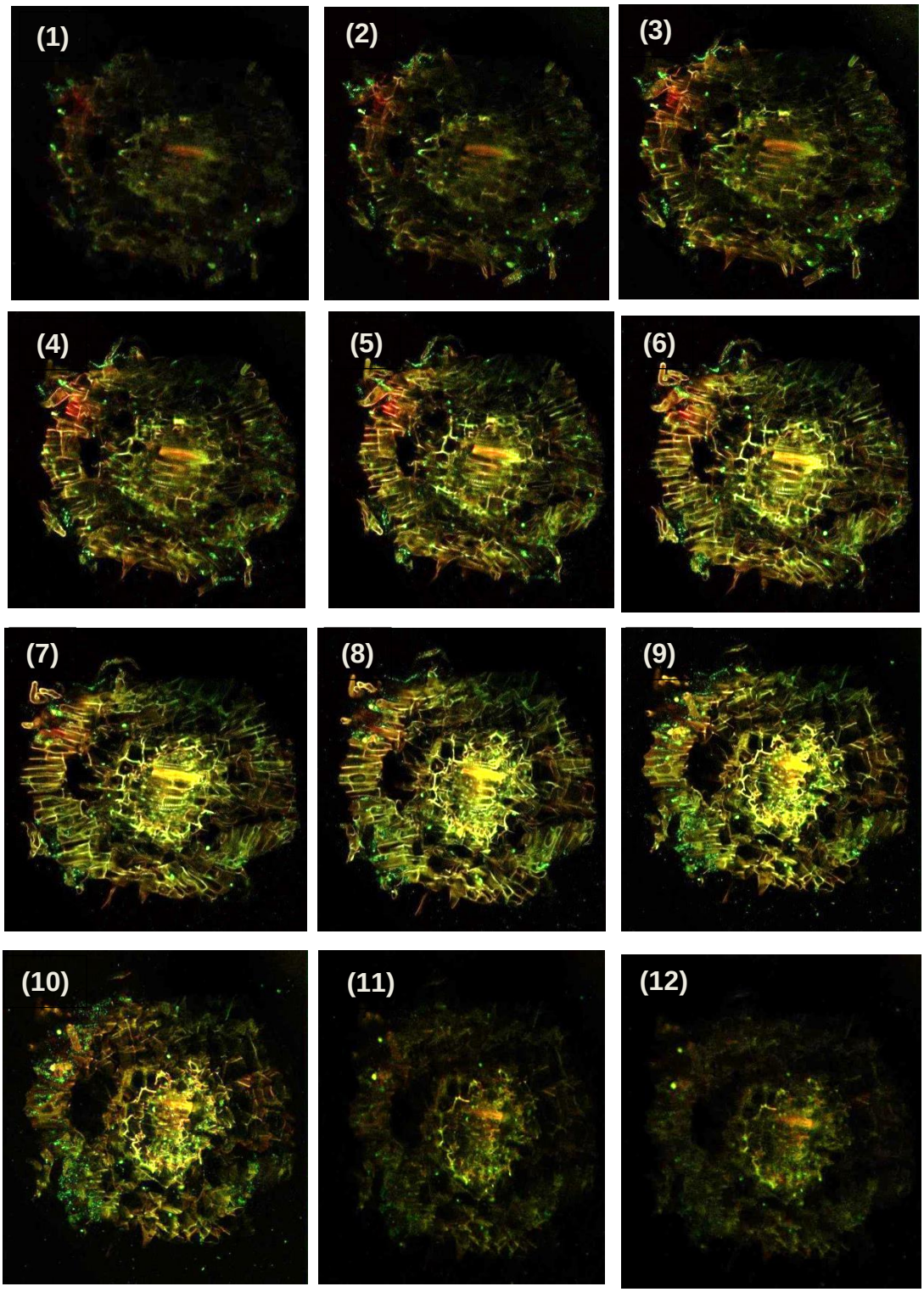



**Figure 3.13: Photographical illustration of CLSM serial optical sections as it was collected along the depth of *B. juncea* root inoculated with bacteria and exposed to Zn. Green and Red patches indicates bacteria and Zn localisation respectively. Sections were numbered according to acquisition sequence along the depth of the root sample**

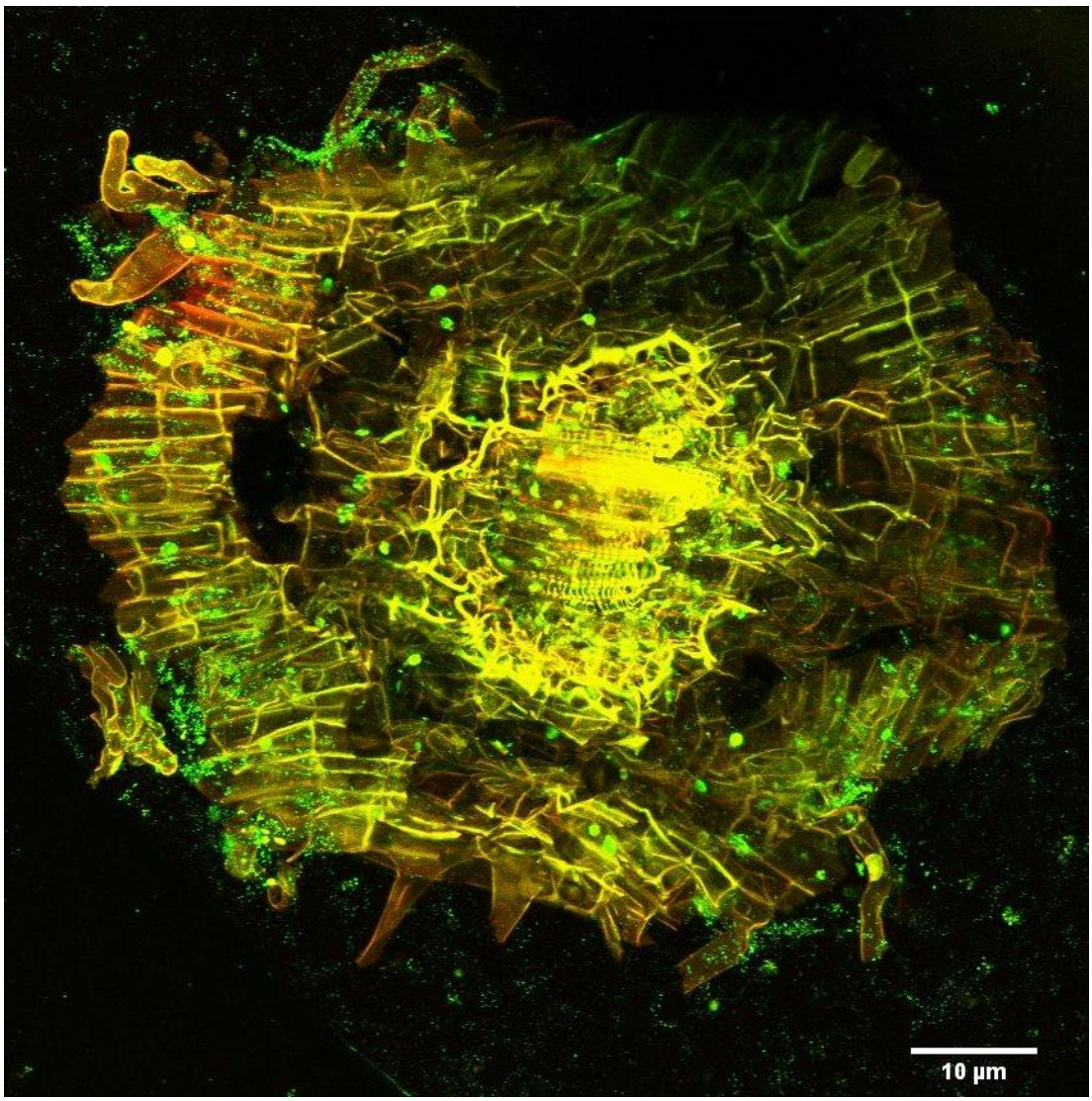



**Figure 3.14: An illustration of the end-product of 3-D reconstruction of stacked optical images (from Fig. 3.12) that were serially collected from the root of *B. juncea* inoculated with bacteria and exposed to Zn. Green and Red patches indicates bacteria and Zn localisation respectively.**

All microscopic imaging was undertaken at the *Centre Optical Instrumentation Laboratory* (*COIL*), University of Edinburgh.

### 3.12. Analysing metal distribution and speciation in plants through synchrotron based X-ray Fluorescence (XRF) imaging and X-ray Absorption Spectroscopy (XAS)

Although the use of fluorophores and fluorescent microscopy in imaging metal distribution in plant biomass has been demonstrated, there are possibilities that the addition of the liquid fluorescent stains to the plant biomass may disturb the nature of metal localisation, influence metal translocation or affect metal concentration in fresh plant biomass. Most importantly, fluorophore based imaging techniques do not have the analytical power to probe the speciation of metals in the plant system. This research therefore further utilised the combination of synchrotron based X-ray Fluorescence (XRF) imaging and X-ray Absorption Spectroscopy (XAS) to study metal distribution and speciation simultaneously, in fresh plant samples. The studies involved transporting live plants growing in pots contaminated with Zn and sectioned plants that were established in contaminated sterile media to Diamond Light Source, UK. Diamond Light Source is the UK's national synchrotron science facility, located at the Harwell Science and Innovation Campus in Oxfordshire. It houses the micro-focus beamline 118 where the analysis were carried out.

The scientific principle behind synchrotron X-ray has been extensively described (Codling, 1992, Kim, 2008, Willmott, 2011). In simple terms, the synchrotron generates high-energy electron beam by using powerful electro-magnets to accelerate electrons to nearly the speed of light (Ungar, 1994). The high-energy electron beam generated is stored in the storage ring and subsequently channeled through auxiliary components such as bending magnets and insertion devices (undulators or wigglers) to produce a very brilliant and highly focused light at the beamlines where they can

be safely used for spectroscopic analysis and imaging (Hendrickson, 2000, Bilderback et al., 2005). Through the use of a tunable monochromator and micro-focusing optical devices, the beamline I18 is a world class research facility that provides high-brightness micron-sized X-ray beam for compositional and spatial analysis of heterogeneous samples at high resolution in conditions close to real life (Mosselmans et al., 2009). A schematic description of the beam optics and sample set up at the experimental hutch of the beamline I18 is presented in Figure 3.15 and 3.16.

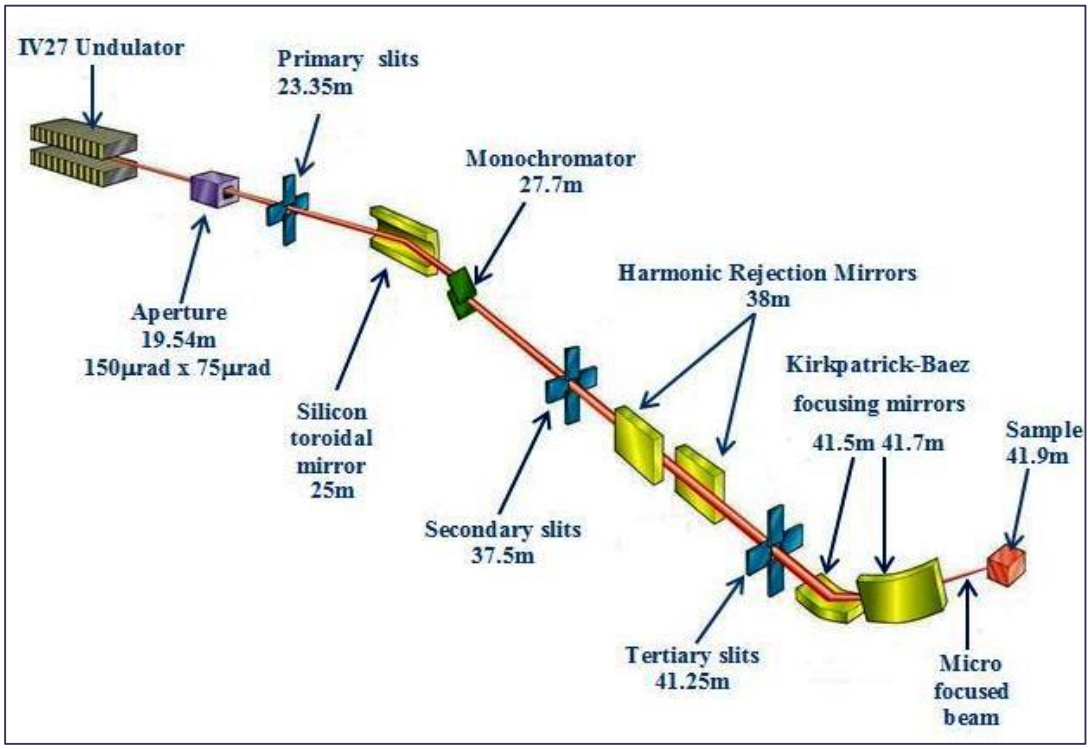



**Figure 3.15: Schematic showing the principal optical elements of the beamline I18 which is based on three mirrors and a liquid nitrogen-cooled double crystal monochromator (Mosselmans et al., 2009)**

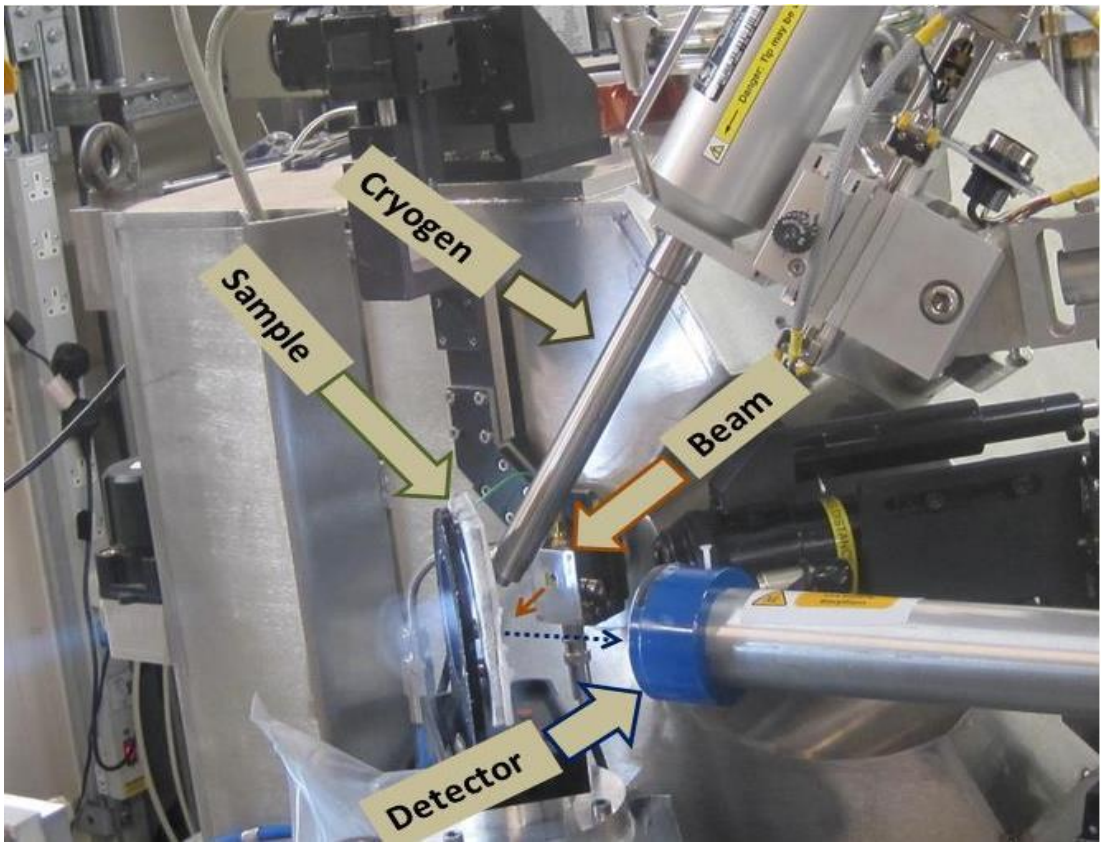



**Figure 3.16: Photographic illustration of experimental setup at the experimental hutch of the beamline I18**

Because roots are the main plant organ to have contact with environmental contaminants and soil bacteria and are the first sites where reaction to metal toxicity takes place (Meagher, 2000, Zhou et al., 2013), roots were analysed for Zn content, distribution and speciation. All samples were immediately cryofixed on the beam sample holder mounted on an x-y-z stage, inclined at an angle of 45° to the incident beam (see Figure 3.16). Synchrotron micro X-ray fluorescence (μXRF) maps of the root samples were collected in fluorescence mode using a nine-element germanium solid state detector. The beamline energy was calibrated using a Zn foil (9661 eV). The collected μXRF data were processed into images using PyMCA 4.4.1, an X-Ray fluorescence analysis software for analyzing μXRF data (Solé et al., 2007).

Points displaying high Zn concentration were selected from the μXRF images for microfocus X-ray Absorption Near Edge Structure (μXANES) analysis. Consecutive spectra from the same point were examined for possible beam damage.

In order to determine the chemical composition of the collected XANES spectra, Zn K-edge μXANES spectra were also collected under similar beam conditions for selected Zn model compounds potentially involved in Zn dynamics within the metal-bacteria-plant-soil system studied (Terzano et al., 2008, Kopittke et al., 2011). The standards of model Zn compounds (Table 3.2) were either freshly prepared using standard preparation protocol designed for preparing compounds for XAS analysis (Salt et al., 1999, Terzano et al., 2008) or purchased.

**Table 3.2 The Zn standards used for XANES analysis**

| Zn standard | Characteristics |
|---|---|
| Zn oxalate | 7.0 mM $Zn(NO_3)_2$ + 70 mM sodium oxalate, pH 7.0 |
| Zn phosphate | 7.0 mM $Zn(NO_3)_2$ + 70 mM sodium phosphate, pH 7.0 |
| Zn histidine | 7.0 mM $Zn(NO_3)_2$ + 80 mM histidine, pH 7.0 |
| Zn cysteine | 7.0 mM $Zn(NO_3)_2$ + 70 mM cysteine, pH 7.0 |
| Zn phytate | 7.0 mM $Zn(NO_3)_2$ + 70 mM phytic acid solution, pH 7.0 |
| Zn polygalacturonate | 7.0 mM $Zn(NO_3)_2$ + 70 mM polygalacturonic acid solution, pH 7.0 |
| Zn formate | 7.0 mM $Zn(NO_3)_2$ + 70 mM formic acid solution, pH 7.0 |
| Zn sulfate, Zn nitrate, Zn citrate, Zn acetate and Zn carbonate | Purchased from Sigma Aldrich |

Solutions standards were held in polythene sample bags while the solid standards were ground homogenized in cellulose and made into pellets for XAS analysis.

The plant samples and standards were scanned through the Zn absorption edge (9630–9850 eV) and μXANES data were processed in Athena, a software designed for XANE analysis (Ravel and Newville, 2005).

In order to model the chemical composition of the samples, the spectra of the Zn standards were subjected to Linear Combination Fitting (LCF) using a least-squares algorithm of the sample μXANES spectrum from 9645.3 to 9725.3 eV. The fractional contribution of each of the analysed standard compounds to the Zn spectrum was assumed to be directly proportional to the fraction of Zn present in that form in the plant root (Terzano et al., 2008). The goodness of the fit was estimated by calculating the residual *R* factor of the fit;

$$R = \Sigma_i \,(\text{experimental-fit})^2 / \Sigma_i \,(\text{experimental})^2 \qquad (3.4)$$

The sums (Σ) are over 103 data points as flattened μ (E). A lower *R* factor represents a better match between the fitted standard spectra and the sample spectrum (Terzano et al., 2008).

Despite the efforts already made to improve the optical capacity of the Beamline I18, it does not possess enough magnification to image bacteria cells. Therefore, a combination of confocal microscopy and synchrotron based X-ray atomic absorption spectroscopy were used to study bacteria-metal co-localisation and speciation in plant samples. This involves acquiring 2 thin consecutive cryo-sectioned samples from the same biomass, subjecting one to confocal fluorescent microscopy and the other (a ‘carbon’ copy of the first) to XAS.

### 3.13. Chapter summary

The Chapter described the materials and methods used for this research and discussed both the scientific rationale and the environmental importance behind their choice. It also presented experimental designs, statistical analysis and scientific methodologies, both existing in literature and newly developed, used throughout the research. The Chapter presented examples of data collected through various experimental methods as illustrations of how the methods work to enhance better understanding of the method described. Subsequent Chapters will focus on presentation and interpretation of data acquired using these methods. Since most of these data Chapters have been written in the form of expanded manuscripts, some of the methods are outlined as a reminder of the basic concepts and justification for their choice.

# 4. The plant growth promoting abilities of *Pseudomonas brassicacearum* and *Rhizobium leguminosarum* on *Brassica juncea* exposed to zinc contamination

This Chapter is based on part of the following manuscript accepted for publication on the 29th of Sep. 2014 in the Journal of Hazardous Materials:

**Gbotemi A. Adediran**, Bryne T. Ngwenya, J. Frederick W. Mosselmans, Kate V. Heal, Barbra A. Havie. Mechanisms behind bacteria induced plant growth promotion and Zn accumulation in *Brassica juncea*.
As the lead author, I performed the experiments and was involved in laboratory analysis. Data analysis and preparation of the first draft of the manuscript was carried out by me. The co-authors provided support and guidance on the scope and design of the study and also specialist laboratory support on XAS analysis. They also contributed to the revision of the manuscript.

## 4.1. Introduction

Phytoremediation, the use of stress tolerant plants for contaminant detoxification, has been identified as a promising sustainable method for cleaning environments contaminated with heavy metals (Salt et al., 1995). Phytoremediation is achieved by growing suitable plants in contaminated environments for a period of time. The process of phytoremediation includes phytovolatilisation, phytostabilisation and phytoextraction. Although less common, phytovolatisation involves the transformation of toxic species of some heavy metals (e.g. Hg) into volatile gases (less toxic species) through the process of transpiration (Sinha et al., 2007).

Meanwhile, phytostabilisation is the immobilisation of metal contaminates at the root of the phytoremediating plant growing in the contaminated soil thus reducing the mobilization of the metal contaminant and lowering the risk of the exposure of metal contamination (Eapen and D'Souza, 2005). Phytoextraction on the other hand

involves the removal of metal from the soil and bioaccumulation of metal contaminants into plant biomass (Jabeen et al., 2009). The phytoextraction of metal contaminants through the use of plants with the ability to accumulate high quantities of toxic metals into the biomass (hyperaccumulators) is often preferable (Ha et al., 2011), since the metal accumulated in the above ground plant biomass can be harvested and concentrated.

For effective and timely phytoextraction, healthy growth of the phytoremediating plant in the metal contaminated soil is required (Kumar et al., 1995). However, hyperaccumulators can be affected by metal toxicity at high soil metal concentrations, exhibiting retarded growth or death before any significant remediation occurs (Ebbs and Kochian, 1997, Lombi et al., 2001, Ma et al., 2009, Babu et al., 2013).

The addition of plant growth promoting bacteria (PGPB) into the plant-contaminant system through seed or soil inoculation has been widely reported to significantly promote the growth of phytoremediating plants exposed to toxic contaminants (Belimov et al., 2005, Ma et al., 2009, Becerra-Castro et al., 2012, Babu et al., 2013). Many species of PGPB have been identified (Belimov et al., 2005, Ma et al., 2011a, Becerra-Castro et al., 2012, Babu et al., 2013) but none has yet gained acceptance as the most efficient in heavy metal microbial-assisted phytoremediation. Inoculating a hyperaccumulator with native endophytic bacteria isolated from the same plant or rhizospheric nutrient fixing and releasing bacteria not necessarily sourced from the same hyperaccumulator have both been suggested as good strategies for efficient microbe-enhanced-phytoremediation (Ma et al., 2011b).

However, the specific mechanisms through which PGPB promote plant growth under heavy metal contamination are still unknown. Mechanisms suggested to date include physiological mediation of deleterious secretion of plant hormones, direct nutrient fixation and release, and change in metals speciation (Khan et al., 2009). The possibility of a single bacteria species performing these roles in a contaminated environment is unrealistic. Therefore developing a dual or multi bacteria hyperaccumulator inoculation system could be the key to the successful implementation of microbial-assisted phytoremediation.

The aim of this study was to explore and compare the plant growth promoting effects of: (i) *Pseudomonas brassicacearum* subsp. *brassicacearum*, an endophytic bacterium isolated from a *Brassica* spp., (ii) *Rhizobium leguminosarum* bv. *trifolii*, a nitrogen fixing rhizobium species, and (iii) the simultaneous combination of the two bacteria, on *Brassica juncea* plants, growing in soil artificially contaminated with Zn. *Brassica juncea* is a well-known accumulator for many heavy metals (Ebbs and Kochian, 1998, Srivastava et al., 2013) and Zn is a widely studied heavy metal contaminant (Long et al., 2011, Gomes et al., 2013). See a more comprehensive justification of the rationale behind the choice of the PGPB, *B. juncea* plant and Zn in Chapter 3.

It was hypothesised that:

(xi) *P. brassicacearum* will promote the growth of *B. juncea* under Zn contamination compared to un-inoculated controls, based on it being a

native bacterium strain isolated from a *Brassica* plant, which should facilitate root colonisation.

(xii) *R. leguminosarum* will enhance *B. juncea* plant growth and survival under Zn contamination compared to un-inoculated controls, based on it being a well-known rhizobacterium of many plant species (both leguminous and non-leguminous) and for its reported ability to promote the growth of other *Brassica* plant species – *Brassica campestris and Brassica napus* (Noel et al., 1996, Schloter et al., 1997).

(xiii) A combination of the two bacteria species will confer multiple plant growth promoting effects under Zn contamination through a combination of their plant growth promoting abilities. i.e. more growth promotion when combination of both bacterial strains are used compared to single bacteria inoculation

## 4.2. Summary of Materials and Experimental methods

### 4.2.1. Materials

The pot experiments used *B. juncea* plant and *Pseudomonas brassicacearum* isolated from a *Brassica* plant and *Rhizobium leguminosarum* isolated from the rhizosphere of a leguminous plant as plant growth promoting bacteria. A standard fertile soil for pot experiments, Scotts Levingston F2+S Seed & Modular growth medium (Vicente et al., 2012), was used (a detailed description of the nature and properties of the soil used in this experiment is presented in Chapter 3).

The source of Zn contamination was Zn sulfate solution. Sulfate was chosen rather than nitrate to avoid the confounding effects of nitrate as a macronutrient.

### 4.2.2. Bacteria toxicity assessment

The Zn tolerance of the selected bacteria was assessed by growing 1 mL pure liquid cultures of each bacterium in 100 mL of standard nutrient broth contaminated with $ZnSO_4.7H_2O$ at 600 and 400 mg Zn $L^{-1}$. Bacteria survival was monitored by evaluating viable cell populations at 24 hours, 48 hours and 144 hours after exposure to Zn by triplicate plating and incubating 0.1 mL of the Zn contaminated media in standard nutrient agar for 2 days. These time periods of exposure were selected to represent the period of seed germination and seedling emergence (3-4 days) for *B. juncea.*

### 4.2.3. Preparation of bacterial inoculants and inoculation of seeds/soil

A colony of each of the bacteria cells was cultured in nutrient broth at 30°C to an exponential growth stage before harvesting, batching and washing. Seeds of *B. juncea* plants were also washed, surface sterilised and dried under a laminar flow hood. For treatments that required single bacteria inoculation, seeds were soaked under aseptic conditions at 30°C for 3 hours in a bacteria suspension containing dry biomass of 0.5 mg $mL^{-1}$ sterile deionised water. Seeds for the treatments with no added bacteria were soaked in sterile deionised water under the same conditions for the same duration. For treatments that required dual microbial inoculation, the *Pseudomonas* species (being a plant endophyte) was administered through seed inoculation while the *Rhizobium* species (being a rhizospheric bacterium) was

administered through soil inoculation in which the sterilised soil was inoculated after the stage of metal stabilisation (see below) with a bacteria suspension of 0.5 mg $mL^{-1}$ dry biomass at the rate of 0.05 mL $g^{-1}$ of soil.

Seed and soil inoculations are standard methods of plant inoculation in bacteria assisted phytoremediation (Burd et al., 2000, Kumar et al., 2008). A multi-process phytoremediation approach using more than one bacteria species, as described in this Chapter, often uses both methods, one for each species (Huang et al., 2005). It is worthy of note that reversing the inoculation system, i.e. administering *Pseudomonas* species as a soil inoculum and *Rhizobium* species as seed inoculum did not have any significant difference on plant growth promotion.

### 4.2.4. Pot experiments

Two pot experiments were conducted in a glasshouse. 0.5 kg of sterilised and dried soil was placed into a 2 liter plastic pot located in a plastic saucer for each experimental replicate. See detailed description of glasshouse conditions in Chapter 3. For the first pot experiment, completely randomized designs of 8 treatments (Table 4.1) were established in duplicate and Zn contamination treatments involved spiking soil in the pots with Zn sulfate solution at the rate of 600 mg Zn $kg^{-1}$ soil dry weight. The pots were then watered with deionised water to field capacity and allowed to stand for 1 week before seeds were planted. Seedlings were established at 2 plants per pot for all treatments and replicates. Plant height above the soil surface was measured weekly for 8 weeks after planting seeds. Above ground and below ground dry biomass per pot was also determined after 8 weeks.

The second growth experiment was conducted in a similar manner to the first but was replicated in 3 pots and Zn contamination treatments were spiked with 400 mg $kg^{-1}$ Zn soil dry weight. A lower dose of Zn was chosen to ensure all treatments had sufficient biomass for further biochemical analysis. Weekly plant heights for this experiment were collected for 6 weeks and dry plant biomass was evaluated after 6 weeks.

**Table 4.1 Description of experimental treatments**

| Treatment | Description |
|---|---|
| B*o* | *B. juncea* plants, un-inoculated, in media soil not contaminated by Zn |
| B*Po* | *B. juncea* plants inoculated with *P. brassicacearum* |
| B*Ro* | *B. juncea* plants inoculated with *R. leguminosarum* |
| B*RPo* | *B. juncea* plants inoculated with *P. brassicacearum* and *R. leguminosarum* |
| BZn | Un-inoculated *B. juncea* plants under Zn contamination |
| B*P*Zn | *B. juncea* plants inoculated with *P. brassicacearum*, under Zn contamination |
| B*R*Zn | *B. juncea* plants inoculated with *R. leguminosarum* , under Zn contamination |
| B*RP*Zn | *B. juncea* plants inoculated with *P. brassicacearum* and *R. leguminosarum*, under Zn contamination |

### 4.2.5. Determination of available nutrients in soil

Plant utilisation of essential plant nutrients from the soil is also a measure of healthy growth in plants (Fageria et al., 2011). Moreover, despite the use of a fertile soil, it is important to investigate if possible reduced growth under metal contamination is due to lack of available essential nutrients. The soil in each of the 3 replicate treatments

from the second glasshouse experiment was sampled at the end of the experiment and two 5 g fresh sub-samples were extracted for total available nitrogen (ammonium-nitrogen and nitrate-nitrogen), available phosphate, potassium, calcium and magnesium. Available nitrogen and phosphate concentrations were determined by automated colorimetry method and the concentrations of available potassium, calcium and magnesium were determined through flame atomic absorption spectroscopy (Carter, 1993).

### 4.2.6. Assessing bacteria colonisation of plant roots

The ability of the bacteria species to survive Zn contamination and colonise the *B. juncea* plant roots until the end of the experiment was investigated. Root strands were sampled in pots 8 and 6 weeks after planting bacteria inoculated *B. juncea* plants in soil spiked with 600 and 400 mg Zn $kg^{-1}$ respectively, and were examined by Scanning Electron Microscopy (SEM). See detailed description of SEM protocol in Chapter 3. Moreover, bacteria colonisation of *B. juncea* roots established for 2 weeks under aseptic laboratory conditions in sterile media contaminated with 400 mg Zn $kg^{-1}$ was examined by fluorescent microscopy using Zeiss Axio Imager 2. Roots established under sterile conditions were analysed to demonstrate that the PGPB possess the ability to survive and colonise *B. juncea* root under Zn contamination.

## 4.3. Statistical Analysis

All treatment means were tested for normal distribution using Anderson-Darling's normality test. All means are of equal variance and are normally distributed (Table 4.2). Means of growth parameters for the two plants were calculated per pot before

the mean from each of the pots were subjected to 1-way Analysis of variance followed by Tukey's HSD test ($p<0.05$) to identify significant differences between pot means. All statistical analyses were conducted using Minitab 16 software (Minitab$^{TM}$ Inc., USA).

Table 4.2(a). Results of Anderson Darling's (AD) normality test for means of bacterial survival assessment in Zn and plant heights from the pot experiments. All treatment means are normally distributed (P-value > 0.05)

| **Treatments** | | **AD-value** | **P-value** |
|---|---|---|---|
| Bacterial survival test in Zn | P-400Zn-24hrs | 0.235 | 0.466 |
| | P-400Zn-48hrs | 0.195 | 0.604 |
| | P-400Zn-144hrs | 0.221 | 0.512 |
| | R-400Zn-24hrs | 0.277 | 0.334 |
| | R-400Zn-48hrs | 0.260 | 0.384 |
| | R-400Zn-144hrs | 0.189 | 0.631 |
| | P-600Zn-24hrs | 0.190 | 0.621 |
| | P-600Zn-48hrs | 0.189 | 0.631 |
| | P-600Zn-144hrs | 0.237 | 0.459 |
| | R-600Zn-24hrs | 0.290 | 0.530 |
| | R-600Zn-48hrs | 0.339 | 0.630 |
| | R-600Zn-144hrs | 0.190 | 0.626 |
| Week 8 plant height first pot experiment | B | 0.272 | 0.316 |
| | BP | 0.260 | 0.485 |
| | BR | 0.233 | 0.557 |
| | BRP | 0.301 | 0.364 |
| | BZn | 0.245 | 0.523 |
| | BPZn | 0.196 | 0.711 |
| | BRZn | 0.196 | 0.713 |
| | BRPZn | 0.251 | 0.510 |
| Week 6 plant height second pot experiment | B | 0.393 | 0.251 |
| | BP | 0.350 | 0.332 |
| | BR | 0.254 | 0.578 |
| | BRP | 0.357 | 0.318 |
| | BZn | 0.363 | 0.421 |
| | BPZn | 0.312 | 0.424 |
| | BRZn | 0.298 | 0.463 |
| | BRPZn | 0.236 | 0.645 |

Table 4.2(b). Results of Anderson Darling's (AD) normality test for means of above and below ground plant biomass from the pot experiments. All treatment means are normally distributed (P-value > 0.05)

| Treatments | | AD-value | P-value |
|---|---|---|---|
| Above ground plant biomass first pot experiment | B | 0.114 | 0.219 |
| | BP | 0.254 | 0.378 |
| | BR | 0.209 | 0.101 |
| | BRP | 0.305 | 0.445 |
| | BZn | 0.147 | 0.197 |
| | BPZn | 0.222 | 0.256 |
| | BRZn | 0.207 | 0.304 |
| | BRPZn | 0.106 | 0.506 |
| Below ground plant biomass first pot experiment | B | 0.212 | 0.331 |
| | BP | 0.334 | 0.415 |
| | BR | 0.204 | 0.191 |
| | BRP | 0.401 | 0.413 |
| | BZn | 0.371 | 0.196 |
| | BPZn | 0.424 | 0.336 |
| | BRZn | 0.412 | 0.164 |
| | BRPZn | 0.116 | 0.406 |
| Above ground biomass second pot experiment | B | 0.314 | 0.419 |
| | BP | 0.254 | 0.578 |
| | BR | 0.219 | 0.711 |
| | BRP | 0.305 | 0.445 |
| | BZn | 0.107 | 0.177 |
| | BPZn | 0.222 | 0.106 |
| | BRZn | 0.357 | 0.164 |
| | BRPZn | 0.246 | 0.606 |
| Below ground biomass second pot experiment | Bo | 0.241 | 0.306 |
| | BP | 0.219 | 0.364 |
| | BR | 0.294 | 0.606 |
| | BRP | 0.351 | 0.295 |
| | B-Zn | 0.23 | 0.235 |
| | BP-Zn | 0.217 | 0.306 |
| | BR-Zn | 0.277 | 0.364 |
| | BRP-Zn | 0.281 | 0.606 |

Table 4.2(c). Results of Anderson Darling's (AD) normality test for means of available nutrients in soil. All treatment means are normally distributed (P-value > 0.05)

| Treatments | | AD-value | P-value |
|---|---|---|---|
| Available N in soil | B | 0.191 | 0.625 |
| | BZn | 0.235 | 0.468 |
| | BPZn | 0.21 | 0.543 |
| | BRZn | 0.189 | 0.631 |
| | BRPZn | 0.194 | 0.609 |
| Available P in soil | B | 0.196 | 0.602 |
| | BZn | 0.226 | 0.503 |
| | BPZn | 0.259 | 0.385 |
| | BRZn | 0.417 | 0.103 |
| | BRPZn | 0.189 | 0.631 |
| Available K in soil | B | 0.208 | 0.549 |
| | BZn | 0.226 | 0.502 |
| | BPZn | 0.264 | 0.372 |
| | BRZn | 0.302 | 0.272 |
| | BRPZn | 0.276 | 0.336 |
| Available Ca in soil | B | 0.418 | 0.102 |
| | BZn | 0.244 | 0.434 |
| | BPZn | 0.203 | 0.572 |
| | BRZn | 0.227 | 0.496 |
| | BRPZn | 0.214 | 0.53 |
| Available Mg in soil | B | 0.203 | 0.569 |
| | BZn | 0.378 | 0.142 |
| | BPZn | 0.192 | 0.621 |
| | BRZn | 0.19 | 0.627 |
| | BRPZn | 0.193 | 0.616 |

## 4.4 Results

### 4.4.1. Bacteria tolerance to Zn toxicity

The viability of *P. brassicacearum*, and *R. leguminosarum* during the period of seed germination and seedling emergence (3-5 days for *B. juncea* plant) was evaluated with 400 and 600 mg $L^{-1}$ of Zn. *P. brassicacearum* (P) was more susceptible to Zn toxicity than *R. leguminosarum* (R) at 24, 48 and 144 hours of exposure under both Zn concentrations (Figure 4.1). However, a significant population of bacterial cells

from the two bacterial strains appeared to be alive after exposure to Zn contamination at similar concentrations to that used in the plant growth experiments (albeit conducted in different media).

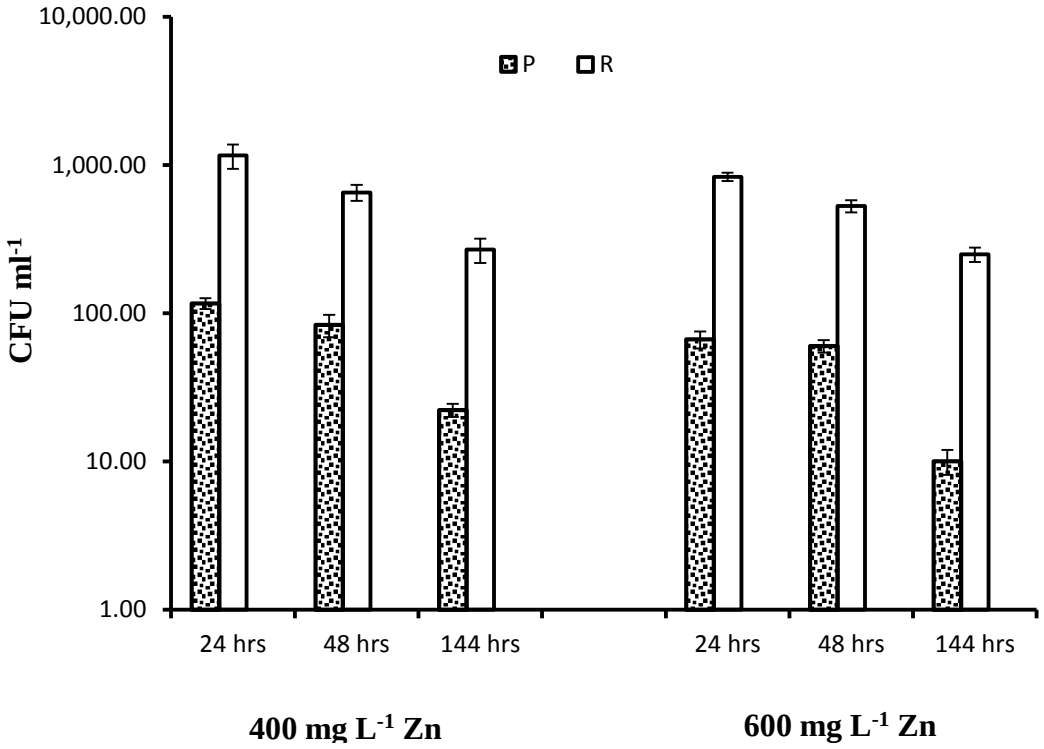



**Figure 4.1: Viability of *P. brassicacearum* (P), and *R. leguminosarum* (R) in 400 and 600 mg Zn $L^{-1}$ over 7 days exposure period. Bars are mean viable cell counts from 3 plates and error bars show standard errors. Figure shows *P. brassicacearum* is more susceptible to Zn contamination but significant amount of bacterial cells from the two strains survived Zn contamination.**

### 4.4.2. Bacteria colonisation of plant roots exposed to Zn

SEM analysis of the rhizoplane of inoculated plant roots shows evidence of bacteria colonisation even at 8 and 6 weeks after planting in soil contaminated with 600 and 400 mg Zn $kg^{-1}$ respectively (Figure 4.2).

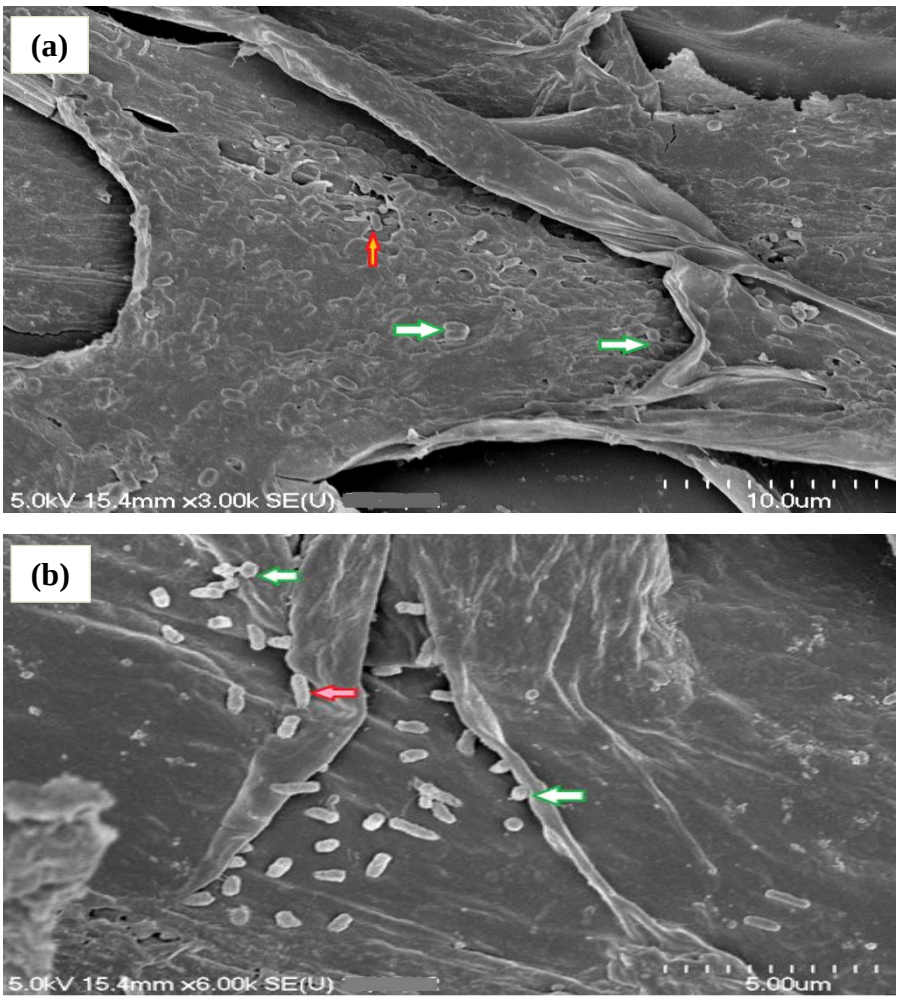



**Figure 4.2: Bacteria at the rhizhoplane of plant inoculated with *R. leguminosarum* and *P. brassicacearum* at (a) 8 weeks after planting in 600 mg Kg $Zn^{-1}$ and (b) 6 weeks after planting in 400 mg Zn $kg^{-1}$. Red and green arrows indicate morphologically different bacteria species. Note that images only demonstrate bacterial colonisation under Zn contamination and there are possibilities that the imaged bacterial cells are not the inoculated strains.**

The experiment conducted under sterile laboratory conditions in sterile media contaminated with 400 mg kg-$^{1}$ Zn gave similar results to the pot experiment (Figure 4.3). The whole root and root cross-section of un-inoculated treatment was completely free of bacterial cells (Figure 4.3a-b). There were however abundant bacterial cells in the whole and sectioned root of treatment inoculated with the two bacterial strains (Figure 4.3c-d).

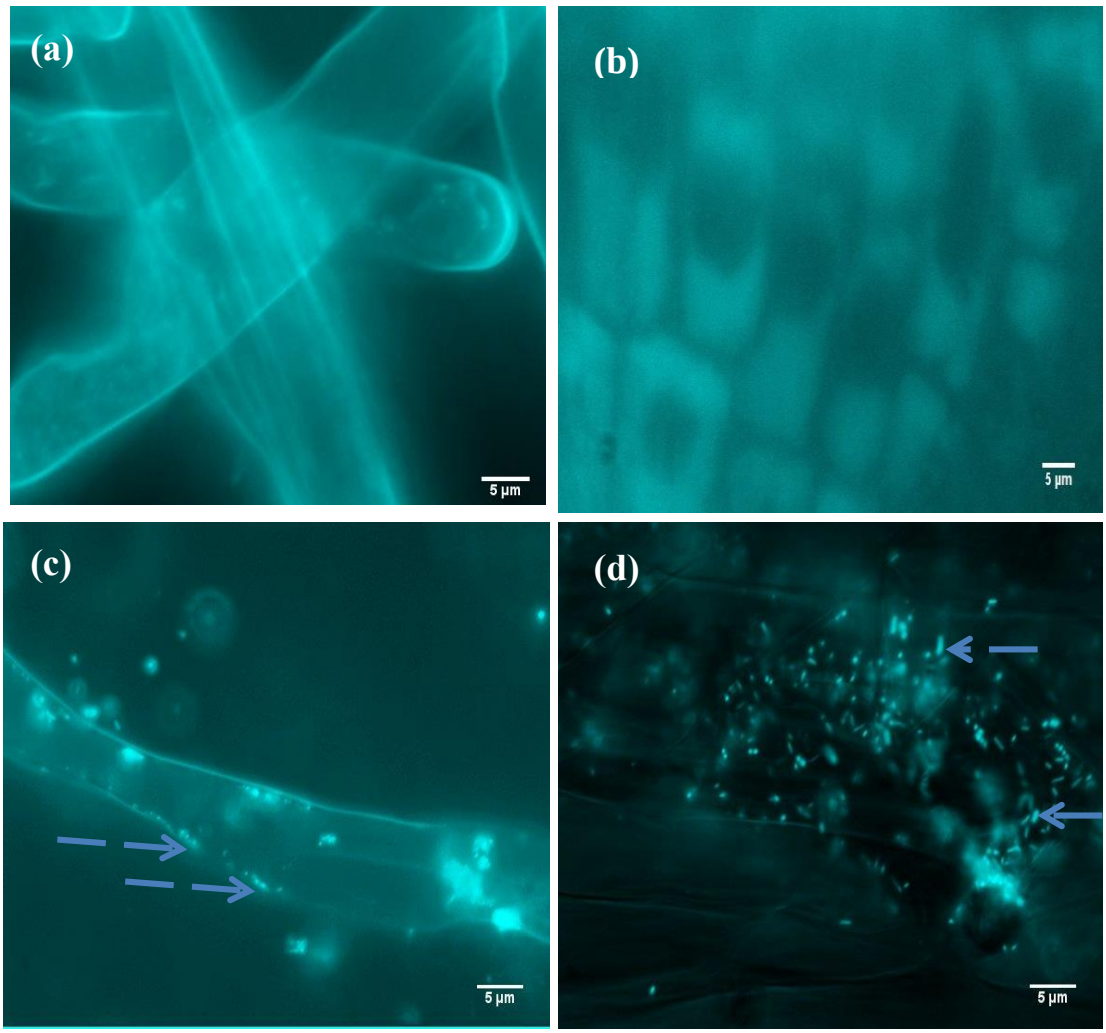



**Figure 4.3: Images showing no bacteria in un-inoculated *B. juncea* (a) root strands and (b) cross-section, and bacteria on (c) root strand and (d) root cross-section of *B. juncea* plant inoculated with *P. brassicacearum* and *R. leguminosarium* at 2 weeks after planting in sterile media contaminated with 400 mg Zn $kg^{-1}$ and maintained in sterile conditions in a sterile growth cabinet. All seeds were surface sterilised with 0.05 M of $NaOCl_4$ and rinsed with sterile deionised water. Seeds for bacterial inoculation were soaked in bacteria-water suspension of $7.5 \times 10^8$ CFU $ml^{-1}$ for 3 hrs and un-inoculated seeds were soaked in sterile water for the same duration. Tissues were stained with 4', 6-diamidino-2-phenylindole (*DAPI*) fluorescent stain and imaged with Zeiss Axio Imager 2. The bacteria (see arrows) are the small fluorescent bodies.**

### 4.4.3. Plant growth promotion by bacteria

The effects of bacterial inoculation on plants growing in uncontaminated soil and in soil contaminated with 600 mg $Kg^{-1}$ Zn were first qualitatively assessed. After 33 days (Figure 4.4), plants grown in the soil contaminated with Zn but without bacteria (BZn) had visibly retarded growth than relative control plants without Zn (Bo).

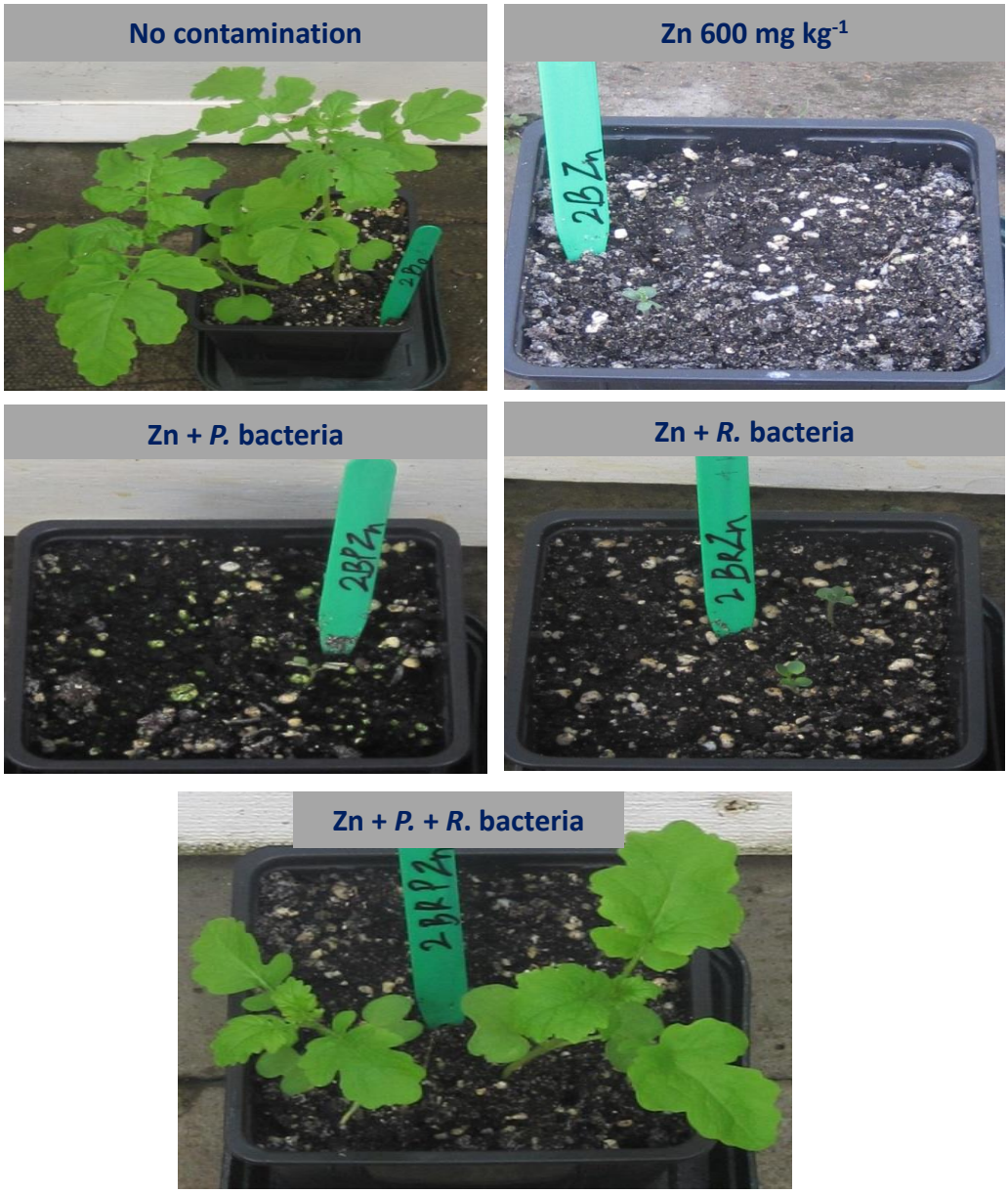



**Figure 4.4: Size and height of *Brassica juncea* plants growing in uncontaminated soil (Bo), soil contaminated with 600 mg $kg^{-1}$ Zn (BZn) under inoculation with *Pseudomonas brassicacearum* (BPZn), *Rhizobium leguminosarum* (BRZn) and combined bacteria (BRPZn) at 33 days after seed planting. Figure shows better growth under Zn in plants inoculated with the two bacterial strains**

These qualitative observations were further confirmed by weekly plant height measurements (Figure 4.5).

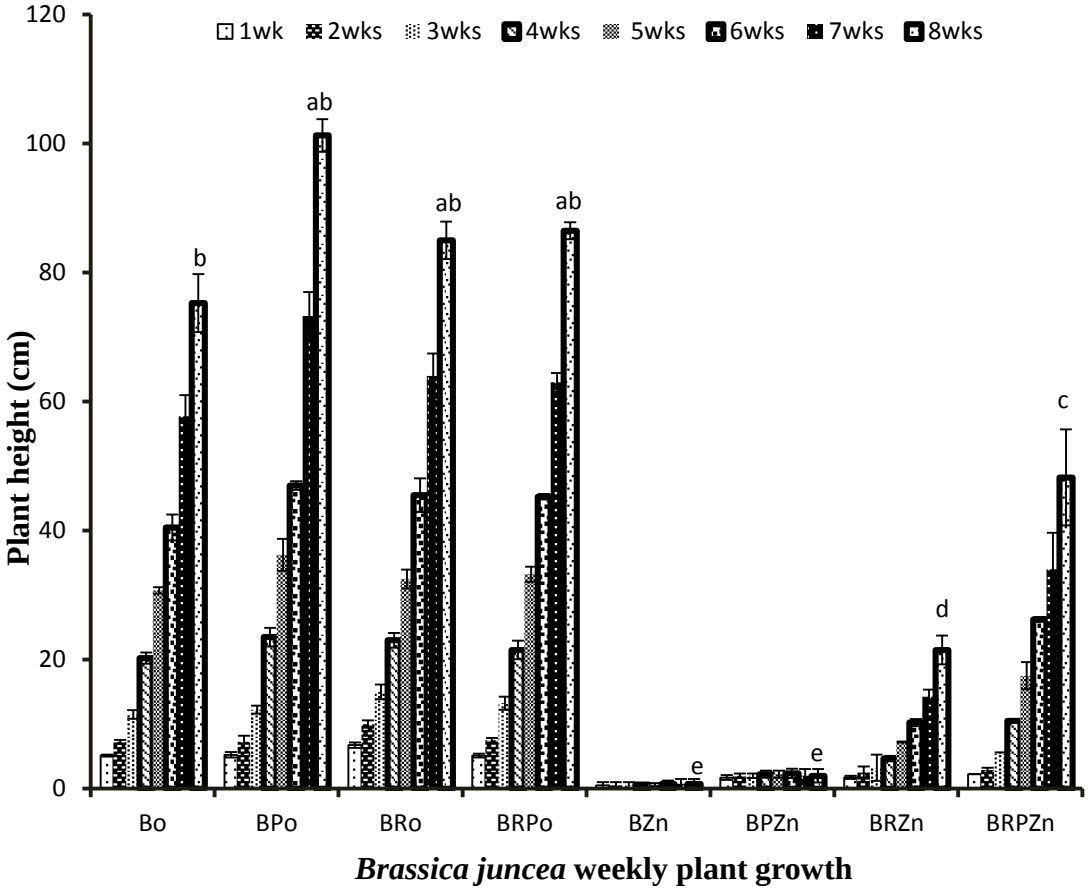



**Figure 4.5: *B. juncea* plant un-inoculated (B), inoculated with *P. brassicacearum* (BP), *R. leguminosarum* (BR) and combinations (BRP) growing in uncontaminated soil (o) and under Zn 600 mg $kg^{-1}$ (Zn). Bars are mean of weekly plant heights from 2 pots. Error bars show standard errors. Different letters indicate significant ($p<0.05$) differences in week 8 plant height between treatments. Figure shows that *R. leguminosarum* and its combination with *P. brassicacearum* significantly promoted plant growth relative to un-inoculated treatments under Zn contamination**

In uncontaminated soil, although the inoculated plants (B*P*o, B*R*o and B*RP*o) appeared to be taller than un-inoculated plants (Bo), their heights were not statistically significantly taller than the (Bo) plants. In soil contaminated with 600 mg $kg^{-1}$ of Zn, however, *B. juncea* was highly susceptible to Zn toxicity (BZn).

Plants grown in the BPZn treatment were statistically significantly shorter compared to the uncontaminated control 8 weeks after planting, although good growth was measured in the first 3 weeks. Meanwhile, BRZn plants showed better growth from 5 weeks after planting. The BRPZn plants gave the best growth results in contaminated soil, with a mean plant height of 48 cm compared to 21 cm for BRZn and 2 cm for BPZn over 8 weeks.

Although the above ground dry biomass was higher in the BRPo treatment, there were no statistically significant differences in above ground dry biomass measured 8 weeks after planting between un-inoculated and inoculated plants grown in uncontaminated soil (Figure 4.6). However, *R. leguminosarum* (BRo) and its combination with *P. brassicacearum* (BRPo) significantly promoted root growth relative to other treatments in uncontaminated soil. When grown in soil contaminated with 600 mg $kg^{-1}$ of Zn, BRZn and BRPZn plants yielded significantly higher above ground dry biomass than BPZn and BZn plants. These plants also have significantly higher root biomass under Zn stress. Most importantly, the above ground biomass in plants inoculated with *R. leguminosarum* (BRZn) and a combination of the two bacteria under metal contamination (BRPZn) was statistically the same with the above ground biomass in plants grown in uncontaminated soil (Bo). Furthermore, *Rhizobium leguminosarum* statistically significantly promoted higher below ground biomass than *Pseudomonas brassicacearum* in uncontaminated soil. The highest below ground plant biomass in uncontaminated soil was, however achieved under dual bacteria inoculation.

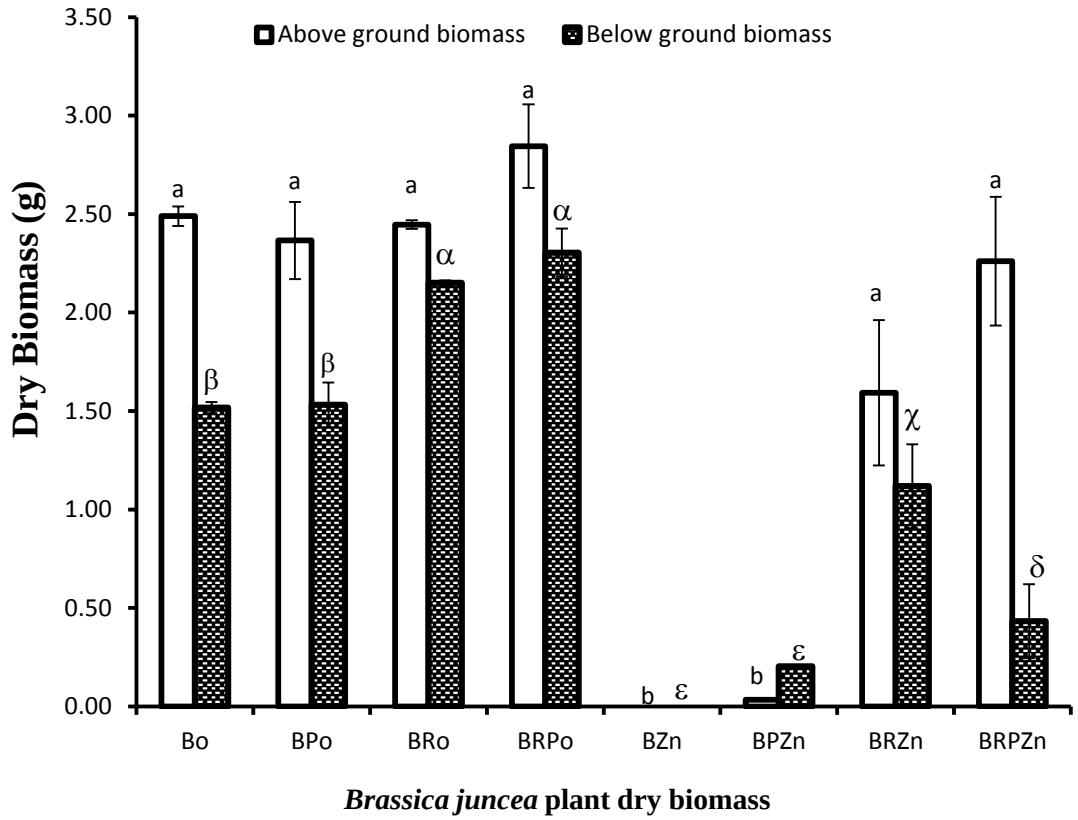



**Figure 4.6: *B. juncea* plant un-inoculated (B), inoculated with *P. brassicacearum* (BP), *R. leguminosarum* (BR) and combinations (BRP) growing in uncontaminated soil (o) and under Zn 600 mg $kg^{-1}$ (Zn). Bars are mean above and below ground plant dry biomass from 2 pots at 8 weeks after seed planting. Error bars show standard errors. Different letters indicate significant ($p<0.05$) difference in above (alphabets) and below (symbols) ground plant dry biomass between treatments. Figure shows that above ground biomass in BRZn and BRPZn treatments are statistically the same with plants in un-contaminated soil an indication of complete shoot recovery under Zn contamination in the inoculated treatments**

Although inoculation of *B. juncea* with *Pseudomonas brassicacearum* (BPZn) appeared to have a slight growth promoting effect on below ground dry biomass under Zn contamination, the effect was not statistically different from that of below ground biomass in un-inoculated plant (BZn). Inoculation of *B. juncea* with *R. leguminosarum* (BRZn) however yielded a significantly higher below ground dry biomass than any other treatments in soil contaminated with 600 mg $kg^{-1}$ Zn.

The second pot experiment conducted under similar experimental conditions but with lower concentration of Zn contamination (400 mg $kg^{-1}$ Zn) and shorter growth period (6 weeks) gave similar results to the one described above (especially under Zn contamination) in terms of growth patterns but the biomass yields were higher, consistent with dose-dependent toxicity. The results of weekly plant growth in *B. juncea* under 400 mg $kg^{-1}$ of Zn contaminations consistently show *R. leguminosarum* (BRZn) as a better plant growth promoter over *P. brassicacearum* (BPZn) and also confirmed dual bacteria inoculation (BRPZn) to have the best growth promoting effects followed by plant inoculation with *R. leguminosarum* (BRZn) (Figure 4.7).

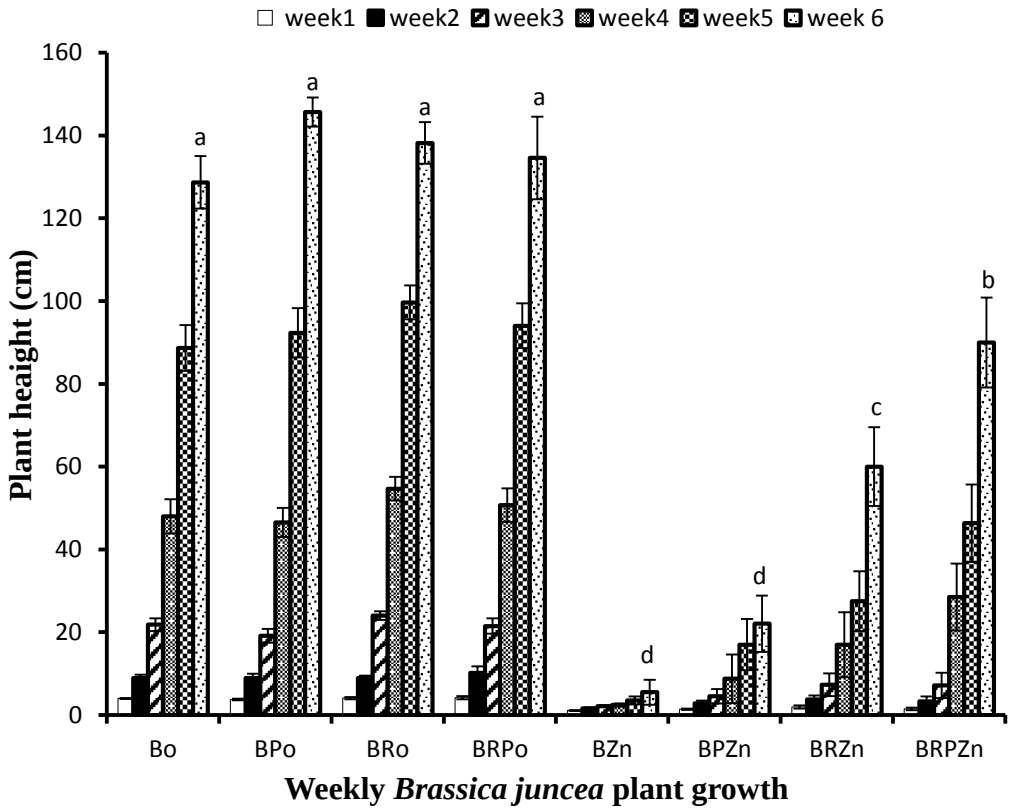



**Figure 4.7: *B. juncea* plant un-inoculated (B), inoculated with *P. brassicacearum* (BP), *R. leguminosarum* (BR) and combinations (BRP) growing in uncontaminated soil (o) and under Zn 400 mg $kg^{-1}$ (Zn). Bars are mean of weekly plant heights from 3 pots. Error bars show standard errors. Different letters indicate significant ($p<0.05$) differences in week 6 plant height between treatments. Figure shows that *R. leguminosarum* and its combination with *P. brassicacearum* significantly promoted plant growth better than un-inoculated treatments under Zn contamination**

Furthermore, higher yields of above and below ground dry biomass were recorded in plants inoculated with *R. leguminosarum* (BRZn) and dual bacteria inoculation (BRPZn) than in plants inoculated with *P. brassicacearum* (BPZn) and un-inoculated plants under soil contaminated with 400 mg kg $Zn^{-1}$ (Figure 4.8).

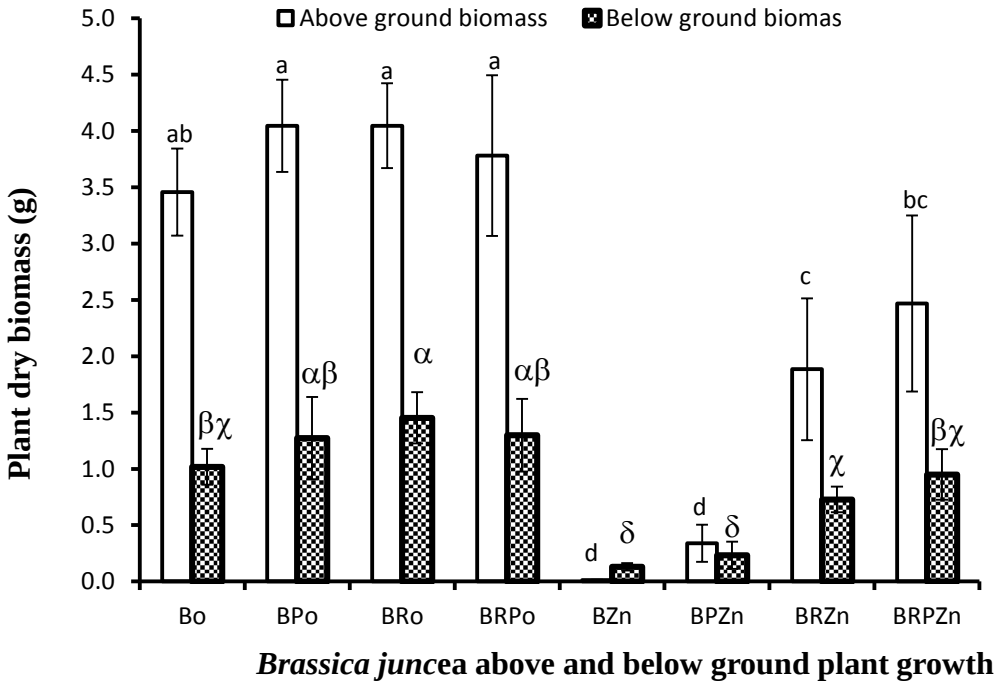



**Figure 4.8: *B. juncea* plant un-inoculated (B), inoculated with *P. brassicacearum* (BP), *R. leguminosarum* (BR) and combinations (BRP) growing in uncontaminated soil (o) and under Zn 600 mg $kg^{-1}$ (Zn). Bars are mean above and below ground plant dry biomass from 3 pots at 6 weeks after seed planting. Error bars show standard errors. Different letters indicate significant ($p<0.05$) difference in above (alphabets) and below (symbols) ground plant dry biomass between treatments. Figure shows that above and below ground biomass in BRPZn treatments are statistically the same with the Bo plant in un-contaminated soil an indication of complete growth recovery of plants under Zn contamination when inoculated with the combination of the two bacterial strains**

Most significantly, both the above and below ground dry biomass in plants inoculated with the two bacteria under metal contamination (BRPZn) were statistically the same with above and below ground dry biomass of un-inoculated plants in uncontaminated soil (Bo) (Figure 4.8).

Utilisation of essential plant nutrients was estimated after the experiment to also serve as an indicator of healthy plant growth under Zn contamination, and most importantly, to investigate if the poor growth observed in the BZn and BPZn plants was due to lack of available essential nutrients under Zn contamination. The amount of available nitrogen, phosphate potassium and calcium in soil under the plants with better growth, in both Zn contaminated and un-contaminated soil (Bo, BRZn and BRPZn) were statistically lower than in soils of the BZn and BPZn treatments (Figure 4.9). Although soil available magnesium content in BPZn treatment was statistically the same with the contents of the BRZn and BRPZn treatments, it was significantly higher in BZn treatments than in BRZn and BRPZn treatments. Overall, there were sufficient amounts of the 5 major essential nutrients in available forms in the soils of the BZn and BPZn treatments where stunted plant growth was observed.

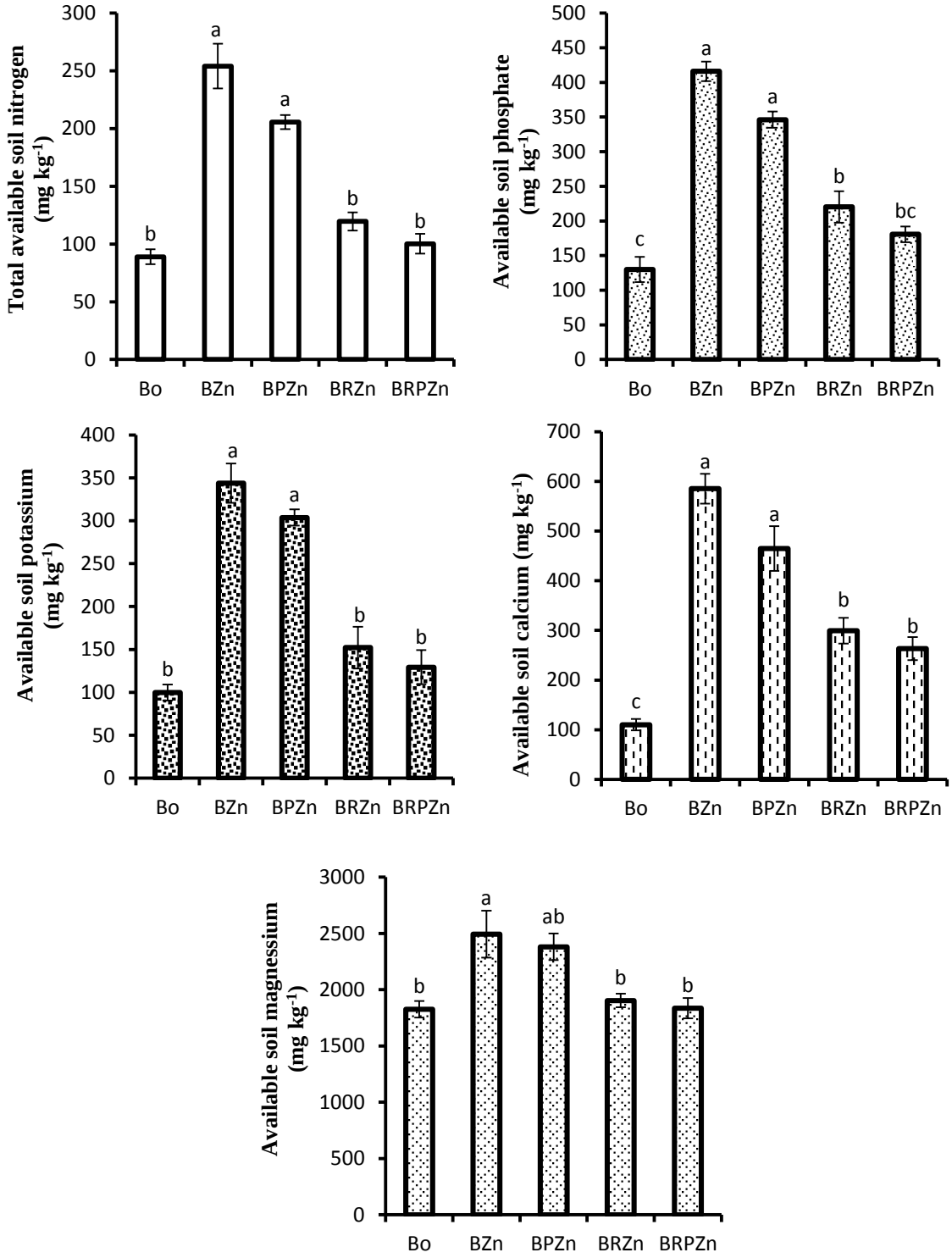



**Figure 4.9: Available nutrients in soils under *B. juncea* un-inoculated (B), inoculated with *P. brassicacearum* (BP), *R. leguminosarum* (BR) and combinations (BRP) in uncontaminated soil (o) and soil contaminated with Zn 600 mg kg$^{-1}$ (Zn) at 6 weeks after seed planting. Bars are mean of total available nitrogen, available phosphate, available potassium, available calcium and available magnesium from 3 pots. Error bars show standard errors. Different letters indicate significant ($p<0.05$) differences between treatments. Figure shows nutrients are in available forms in soil under BZn and BPZn plants even at 6 weeks after planting and the poor growth in these treatments was not due to lack of sufficient nutrients in available forms under Zn contamination**

### 4.5. Discussion

The major findings from this study are highlighted as follows:

(i) The bacterial strains, *P. brassicacearum* and *R. leguminosarum* exhibited resistance to Zn toxicity and are able to colonise *B. juncea* roots under Zn contamination

(ii) Plant growth promoting abilities of the bacterial strains were not statistically significant in soil without Zn contamination

(iii) The plant growth promoting ability of *R. leguminosarum* was statistically significantly different from that of *P. brassicacearum* under Zn contamination

(iv) Inoculation of plants with the combination of the two bacterial strains had the best plant growth promoting effects on plants under Zn contamination.

(v) Poor growth under Zn contamination in plants without bacterial inoculation and plants inoculated with *P. brassicacearum* was not due to lack of essential nutrient availability for plant use in the treatments.

Resistance of bacteria to metal toxicity has been observed in many bacteria species and the mechanism of bacterial resistance to high metal contamination in the environment has been well researched (Gadd and Griffiths, 1977, Nies, 1999, Bruins et al., 2000). Metal resistance in bacteria has been shown to be controlled by the bacterial plasmids which carry the genes for resistance to many metals (Trevors et al., 1985, Silver and Phung, 1996). Efflux of metal ions outside the bacterial cell wall, metallothioneins sequestration and reduction of metal ions to less toxic forms have been identified as possible mechanisms for bacterial survival under metal

toxicity (Choudhury and Srivastava, 2001, Saluja and Sharma, 2013). Although the specific toxicity resistance mechanism of the bacterial strains used in this study was not tested, other studies have suggested that PGPB like *R. leguminosarum* possess well developed plasmid-encoded catabolic genes which may be involved in metal resistance as well as in plant nodulation (Spaink et al., 1987, Oresnik et al., 1998). Moreover, the tolerance of *Psudomonas putida* to Cd toxicity is also thought to involve secretion of oxidative stress protective proteins and the P-type ATPase, CadA2 associated to $Cd^{2+}$ efflux (Miller et al., 2009).

Apart from exhibiting a significant level of resistance to Zn contamination the bacterial strains were also able to colonise plant roots under Zn contamination. Although there are significantly more studies focusing on appraising the beneficial effects of bacteria to plants than the beneficial effects of plants to bacteria, there are strong lines of evidence that plants encourage and enhance bacteria colonisation and survival in uncontaminated soils as well as in soils contaminated with metals (Marilley et al., 1998, Kozdrój and van Elsas, 2000). The rhizosphere, the soil environment attached to the plant root system (Smalla et al., 2006) has been widely identified as the hot spot of abundant bacteria population and activity due to the presence of root exudates (el Zahar Haichar et al., 2008, Compant et al., 2010). Plant root exudates are primarily carbon containing compounds, known collectively as rhizodeposits that are derived from the products of photosynthesis (Bertin et al., 2003, Dennis et al., 2010). Rhizodeposits include a wide variety of bacteria sustaining carbonaceous compounds like sugars, amino acids, organic acids, fatty acids, sterols, vitamins and enzymes (Dennis et al., 2010). In other research, it has been shown that

roots under metal contamination exude carbonaceous compounds as part of their defense mechanism against the effect of metal toxicity (Kozdrój and van Elsas, 2000, Barcelo and Poschenrieder, 2002). Apart from the inherent metal tolerant ability of the bacteria species, it is therefore very likely that *B. juncea* plant roots positively contribute to the survival of active bacteria cell populations imaged at 2, 6, and 8 weeks after planting in environment contaminated with 400 and 600 mg $Kg^{-1}$ Zn. Moreover, it is not all the Zn added to the soil that will be bioavailable as some will be adsorbed to the soil minerals or precipitate into compounds of lower solubility and thus lower toxicity in the soil (Martınez and Motto, 2000, Heike, 2004). It is also therefore likely that the abundant bacterial survival and colonisation of roots under Zn contamination was favoured by the capacity of the soil to reduce metal toxicity.

Furthermore, in the two glasshouse experiments, there were no statistically significant differences in plant growth parameters between the inoculated treatments and the treatments without bacterial inoculation in soil without Zn contamination. The absence of any apparent growth promotion effects due to bacterial inoculation could be due to the use of a soil that has been formulated to meet plant nutrient needs to ensure plants do not suffer from nutritional stress on top of metal-induced stress. However, the stunted growth in the un-inoculated (BZn) plants with signs of yellow leaf colouration in plants inoculated with *P. brassicacearum* (BPZn) under 600 mg kg-1 of soil Zn contamination are typical symptoms of Zn toxicity in plants (Ebbs and Kochian, 1997).

Results from the repeated experiments clearly showed that the two bacterial strains possess the ability to promote plant growth under Zn contamination, with the *R. leguminosarum* being a better growth promoter. Although the phenomenon of better growth under metal contamination in plants inoculated with bacteria has been widely observed and reported (Burd et al., 2000, Rajkumar and Freitas, 2008) the specific process through which bacteria promote growth under metal contamination is still un-clear. For example, nitrogen fixation, phosphate solubilisation and overall improvement of plant nutrition by bacteria in soil contaminated with metal, have been widely suggested as the main mechanism of plant growth promotion by the PGPB (Mayak et al., 2004a, Khan, 2005, Rajkumar et al., 2009).

However, the lack of apparent growth promotion by bacteria in un-contaminated soil suggests addition of more nutrients for example through nitrogen fixation, is of little effect in this study. Most importantly, analysis of nutrient availability in the soil of treatments under Zn contamination clearly shows that the highest amount of essential nutrients in available forms are in BZn and BPZn treatments where poor growth were recorded. The BZn and BPZn plants could not grow despite the availability of sufficient essential plant nutrients in the soil due to Zn toxicity. This therefore confirms that susceptibility to Zn toxicity and growth promotion under Zn contamination does not have anything to do with improvement in availability of essential nutrients especially in fertile soils and could therefore not be the mechanism of plant growth promotion by bacteria as observed in BRZn and BRPZn in this study.

Moreover, plants under metal contamination have been observed to suffer poor growth from metal toxicity indirectly through the secretion of excessive amount of ethylene in plants (Lynch and Brown, 1997, Burd et al., 1998). PGPB like *P. brassicacearum* and *R. brassicacearum* has been reported to have the ability to produce the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, metabolise ACC; a precursor of ethylene in plants and thus help reduce the level of ethylene secretion to optimum in plants under metal toxicity (Belimov et al., 2001, Belimov et al., 2005). This mechanism has however been reported to be possible but only a passive effect of PGPB in promoting plant growth under metal contamination (Arshad et al., 2007, Glick et al., 2007). Moreover, excessive amount of ethylene gas secretion is only one of the possible indirect effects of metal toxicity as condensation of cellular chromatin materials, disruption of cortical cell organelles in plant root, dilation of nuclear membrane and collapse of the vacuole has been reported in other works to be direct toxic effects of metals in plant (Hall, 2002, Rout and Das, 2003). Although the mechanism through which the bacterial strains promoted *B. juncea* growth under Zn contamination is not known at this stage, it is more likely that bacteria directly reduces the toxicity of Zn to plants through changes in Zn bio-toxicity or through other means in the plant root rather than improvement of plant nutrition or moderation of ethylene gas secretion in plants.

Moreover, the inoculation of the *B. juncea* plant with the two bacteria consistently gave the best plant growth promoting under Zn contamination. Most importantly, results of dry biomass under Zn contamination in plants inoculated with the two

bacteria were statistically the same with the growth of un-inoculated *B. juncea* in un-contaminated soils. This shows inoculation of *B. juncea* with a combination of *R. leguminosarum* and *P. brassicacearum* is a potential bio-enhancement technology for helping *B. juncea* recovers full growth as well achieving optimum phytoremediation efficiency under Zn contamination. It is important to note that growth promotion observed under dual bacteria inoculation is more than the addition of growth promotion effects of individual bacteria. This suggests that the two PGPB may be performing specific but synergistic roles which at this stage are not very clear. Dual inoculation of cowpea plants with an arbuscular mycorrhizal fungi and nitrogen-fixing rhizobium bacterium has been reported to significantly increase Zn tolerance in plants (Saleh and Saleh, 2006). Thus *R. leguminosarum* may help reduce the metal toxicity which then enhances the plant growth promoting ability of the *P. brassicacearum* under Zn contamination. Using two bacteria species of contrasting biochemical properties in inducing toxic metal tolerance has not been reported.

### 4.6. Conclusion

It can therefore be concluded that the plant growth promoting ability of *Pseudomonas brassicacearum*, the bacteria species isolated from the Brassica plant, is very limited under Zn contamination. The inoculation of *B. juncea* with *Rhizobium leguminosarum*, however promoted plant growth under Zn contamination significantly better than *Pseudomonas brassicacearum* inoculations as well as in comparison to un-inoculated plants exposed to Zn contamination.

Although it was hypothesised that inoculation of *Brassica juncea* with a native bacteria strain (*Pseudomonas brassicacearum)* will yield a better plant growth than

in un-inoculated plants under Zn contamination, this was not so. Results from this research shows that a bacterium that was not isolated from a Brassica plant had a better growth promoting effects on a Brassica plant under Zn contamination, than the native bacterial strain. Inoculating plants with native bacteria species may not therefore have significant plant growth promoting effects under Zn contamination despite the anticipated beneficial bacteria/plant compatibility.

As hypothesised, the combinations of both bacteria species promoted plant growth significantly more than any other treatments. This clearly shows that different bacteria species may be performing different plant growth promoting roles under Zn contamination, and these different roles may have synergistic effects that may be further explored to confer tolerance to high soil Zn concentrations and as well enhance Zn phytoremediation. Moreover, it is important to assess if bacteria induced plant growth promotion as reported in this Chapter will also lead to increased Zn bioaccumulation and soil Zn phytoremediation before the use of bacteria in metal phytoremediation could be recognised as a potential technology for remediating metal contaminated soil.

Chapter 5 will examine the mechanism behind plant growth promotion by bacteria in plants under metal contamination based on results from synchrotron based XAS analysis of metal biochemistry in the bacteria-plant- metal system of live plants. It will also investigate if enhanced plant growth under inoculation with the bacterial strains will lead to increase in Zn bioaccumulation and soil Zn phytoremediation in soil contaminated with Zn.

Chapter 5

# 5. Mechanisms behind bacteria induced plant growth promotion, Zn bioaccumulation and phytoremediation in *Brassica juncea*

This Chapter is based on part of the following manuscript accepted for publication on the 29th of Sep. 2014 in the Journal of Hazardous Materials:

**Gbotemi A. Adediran**, Bryne T. Ngwenya, J. Frederick W. Mosselmans, Kate V. Heal, Barbra A. Harvie. Mechanisms behind bacteria induced plant growth promotion and Zn accumulation in *Brassica juncea*.
As the lead author, I performed the experiments and was involved in laboratory analysis. Data analysis and preparation of the first draft of the manuscript was carried out by me.
The co-authors provided support and guidance on the scope and design of the study and also specialist laboratory support on XAS analysis. They also contributed to the revision of the manuscript.

## 5.1. Introduction

The growth of plants exposed to toxic metals has been reported to be enhanced by inoculating the plants with plant growth promoting bacteria (PGPB) but the mechanisms behind this process remain debatable. Some of the mechanisms suggested include PGPB simulation of plant growth by directly providing plants with fixed nitrogen, phosphate, sulfate and micro nutrients like iron and manganese in metal contaminated environment (Mayak et al., 2004a, Khan, 2005, Rajkumar et al., 2009). Provision of growth enhancing phytohormones like the auxins (indole-3-acetic acid), cytokinins and gibberellin by PGPB to plants has also been suggested as possible mechanisms behind better growth in PGPB inoculated plants under metal contamination (Khan, 2005, Rajkumar and Freitas, 2008, Ma et al., 2011a).

Moreover, PGPB secretion of the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase in metal contaminated environments to prevent excessive

production of ethylene to a level that may be deleterious to plant root growth has also been suggested as another mechanism (Nie et al., 2002, Glick, 2003). Furthermore, protection of plants against fungal, bacterial, and viral diseases as well as insect and nematode pests has also been suggested as possible beneficial effects of PGPB through which plant growth is enhanced under metal contamination (Zhuang et al., 2007, Ma et al., 2011a).

However, studies conducted in metal contaminated media where the essential plant nutrients and growth hormones are provided and under experimental conditions free of plant pests and diseases have also reported reductions in metal toxicity in plants inoculated with PGPB. These observations have been linked to possible changes in metal speciation induced by bacteria and this is being regarded as a major plant growth promoting mechanism (Burd et al., 2000, Wu et al., 2006, Madhaiyan et al., 2007). Although these studies provided valuable insights towards the understanding of the roles of PGPB in metal sequestration, they mostly utilised destructive analytical techniques (like chemical extraction of hormones, enzymes and nutrients from bacteria and plants) to study bacteria, metal, and plant as three different units (Wu et al., 2006, Rajkumar and Freitas, 2008, Khan et al., 2009) and the likelihood of metal speciation changing during these destructive sample preparation method are very high (Feldmann et al., 1999, Hammer and Keller, 2002, Amaral et al., 2013). The bacteria-metal-plant system is however a dynamic system that needs to be studied as a unit under minimal disturbance in order to properly identifies the roles of PGPB in metal translocation, sequestration and possible changes in speciation in plants.

This Chapter therefore examines possible bacteria induced changes in Zn distribution and speciation in live *B. juncea* plants as possible mechanisms behind bacteria induced plant growth promotion under Zn contamination. It also evaluates the contribution of increased plant growth to Zn bioaccumulation and soil Zn remediation. *B. juncea* plants growing on soils contaminated with 400 mg $kg^{-1}$ Zn for 6 weeks were studied using a combination of synchrotron based μXRF imaging and μXANES analysis.

It was hypothesised that:

(i) *Pseudomonas brassicacearum* and *Rhizobium leguminosarum* having been established to have plant growth promoting abilities under Zn contamination will induce changes in metal sequestration and speciation in *B. juncea* plants.

(ii) The nature of Zn accumulation, distribution and speciation in *B. juncea* root inoculated with *Pseudomonas brassicacearum* will be different from *B. juncea* root inoculated with *Rhizobium leguminosarum* due to the differences in plant growth promoting abilities of the two bacteria under Zn contamination.

(iii) There will be higher Zn bioaccumulation and soil Zn remediation in plants inoculated with the two bacteria than in plants under single bacterial inoculations due to higher plant growth promotion observed in plants under dual bacteria inoculation.

## 5.2. Experimental

### 5.2.1. Experimental treatments

A standard fertile soil for pot experiments, Scotts Levingston F2+S Seed & Modular growth medium (Vicente et al., 2012), was used (See detailed soil properties and nutritional composition in Chapter 3). The soil was sterilised and dried in an autoclave to ensure it was free of insect and nematode pests, bacterial and viral diseases. Pot experiments were conducted in a glasshouse for 6 weeks, with XAS analysis carried out at week 5. 0.5 kg of soil was placed into a 2 litre plastic pot located in a plastic saucer for each experimental replicate. A completely randomized design of 6 treatments (Table 5.1) was established in triplicate pots.

**Table 5.1: Description of experimental treatments**

| Treatment | Description |
|---|---|
| Bo | *B. juncea* plants, un-inoculated, in soil not contaminated by Zn |
| -Zn | Zn contaminated soil without plants |
| BZn | Un-inoculated *B. juncea* plants under Zn contamination |
| BPZn | *B. juncea* plants inoculated with *P. brassicacearum*, under Zn contamination |
| BRZn | *B. juncea* plants inoculated with *R. leguminosarum* , under Zn contamination |
| BRPZn | *B. juncea* plants inoculated with *P. brassicacearum* and *R. leguminosarum,* under Zn contamination |

Zn contamination treatments involved spiking soil in the pots with Zn sulphate solution at the rate of 400 mg Zn $kg^{-1}$ soil dry weight. The same volumes of deionised water were added to the controls. The pots were then watered with

deionised water to field capacity and allowed to stand for 1 week before seeds were planted. Seeds were planted at the rate of 5 per pot. Seedlings were weeded 5 days after emergence, to leave 2 plants in each pot. To prevent water stress the soil was kept moist throughout the experiment. Plant heights above the soil surface, and above and below ground dry biomass per pot were determined after 6 weeks.

### 5.2.2. Zn phytoremediation and bioaccumulation analysis

Zn phytoremediation and accumulation by the plants in different treatments was assessed. Soils sampled from the pots at the end of the experiment were extracted using a modified BCR sequential extraction method to determine exchangeable (readily bioavailable), reducible and oxidisable Zn fractions. Briefly, acetic acid (0.11 mol $l^{-1}$), hydroxylamine hydrochloride (0.5 mol $l^{-1}$), hydrogen peroxide (8.8 ml $l^{-1}$) and ammonium acetate (1.0 mol $l^{-1}$) were used to obtain exchangeable, reducible and oxidisable Zn fractions in 1g of soil respectively (Rauret et al., 1999). The residual Zn content was extracted in aqua regia, a 1:3 mixture of nitric and hydrochloric acids, for 3 h at 110°C (Rauret et al., 2000). Total Zn concentrations in the harvested roots and shoots were determined using wet acid digestion methods (Lowther, 1980) in two sub-samples of the combined harvested and dried above ground and below ground biomass per pot. Blanks from the extracts and digests were analysed and subtracted from analytical results. The background soil Zn concentrations in the uncontaminated control pots were also deducted from the results. Zn concentrations in the soil and plant extracts and digests were determined by ICP-OES (Perkin Elmer Optima 5300 DV) and the results reported as the mean of the two sub-samples of each material corrected to dry weight.

Soil Zn phytoextraction efficiency was estimated by calculating Zn removal percentage based on plant Zn accumulation and soil Zn reduction.

(i) Based on plant Zn accumulation, percentage soil Zn removed was calculated as (ZnP/ZnSoil) x100. Where ZnP is the total Zn in plant biomass (below and above ground) and ZnSoil is the concentration of Zn added to soil.

(ii) Based on comparison of Zn concentration in soil, percentage Zn removal was calculated as $[(C_o - C_f)/C_o]$ x100 where C*o* and $C_f$ are total soil Zn content in contaminated pots without plants and in contaminated pots under phytoremediation respectively (Bennett et al., 2003).

Zn yield in above and below ground biomass was estimated by multiplying Zn concentration in dry biomass with dry biomass weight. The Zn bioaccumulation factor (BF) was calculated as: $BF = C_p/C_s$, where $C_p$ is Zn concentration in the total (above and below ground) harvested dry plant biomass (mg $kg^{-1}$) and $C_s$ is the total Zn concentration (addition of the sequentially extracted fractions and residual Zn concentration) in the contaminated soil (mg $kg^{-1}$) at the end of the experiment (Zhao et al., 2003). Zn translocation efficiency from the root to the shoot biomass of the plant was calculated as the translocation factor (TF) for each plant: $TF = C_{shoot}/C_{root}$, where $C_{shoot}$ and $C_{root}$ are the Zn concentrations in the harvested plant above ground and below ground biomass, respectively (Marchiol et al., 2004).

### 5.2.3. Synchrotron-based X-ray spectroscopic analysis

Live plants were transported to Diamond Light Source, Didcot, UK, at 5 weeks after planting and analysed on the microfocus beamline I18 (Mosselmans et al., 2009). We focussed on analysing Zn distribution and speciation in roots, on the basis that roots

are the main plant organ to have contact with environmental contaminants and soil bacteria and are where the first reaction to metal toxicity takes place (Meagher, 2000, Zhou et al., 2013). Root samples were excised, rinsed with sterile deionised water and immediately cryofixed on the beam sample holder mounted on an x-y-z stage, inclined at an angle of 45° to the incident beam. The beamline energy was calibrated using a Zn foil (9661 eV). Synchrotron micro X-ray fluorescence (μXRF) data of the root samples were collected in fluorescence mode using a nine-element germanium solid state detector. The collected μXRF data were processed into images using PyMCA 4.4.1 (Solé et al., 2007). Points displaying high Zn concentration were selected from the μXRF images for microfocus X-ray Absorption Near Edge Structure (μXANES) analysis. Zn K-edge μXANES spectra were also collected under similar beam conditions for selected Zn model compounds potentially involved in Zn speciation within the metal-bacteria-plant-soil system studied (Terzano et al., 2008, Kopittke et al., 2011). Standards of model Zn compounds (Table 5.2) were freshly prepared or purchased.

**Table 5.2: Standard Zn compounds used for XAS analysis**

| Zn standard | Characteristics |
|---|---|
| Zn oxalate | 7.0 mM $Zn(NO_3)_2$ + 70 mM sodium oxalate, pH 7.0 |
| Zn phosphate | 7.0 mM $Zn(NO_3)_2$ + 70 mM sodium phosphate, pH 7.0 |
| Zn histidine | 7.0 mM $Zn(NO_3)_2$ + 80 mM histidine, pH 7.0 |
| Zn cysteine | 7.0 mM $Zn(NO_3)_2$ + 70 mM cysteine, pH 7.0 |
| Zn phytate | 7.0 mM $Zn(NO_3)_2$ + 70 mM phytic acid solution, pH 7.0 |
| Zn polygalacturonate | 7.0 mM $Zn(NO_3)_2$ + 70 mM polygalacturonic acid solution, pH 7.0 |
| Zn formate | 7.0 mM $Zn(NO_3)_2$ + 70 mM formic acid solution, pH 7.0 |
| Zn sulfate, Zn nitrate, Zn citrate, Zn acetate and Zn carbonate | Purchased from Sigma Aldrich |

Standard solutions were held in polythene sample bags while the solid standards were ground, homogenized in cellulose and made into pellets for analysis. The root samples and standards were scanned through the Zn absorption edge (9630–9850 eV). Consecutive spectra from the same point were examined for possible beam damage. For each of the treatments, 6 good μXANES spectra were selected, merged and subjected to further analysis. μXANES data were processed in Athena (Ravel and Newville, 2005). All μXANES spectra collected from the samples and standard were properly normalised and aligned. Linear Combination Fitting (LCF) using a least-squares algorithm of the sample μXANES (from 9645.3 to 9725.3 eV) was performed using the spectra of the standards. For all μXANES fitting, the weights of the standard's spectra in the sample's spectra were not forced to sum to 1. Although all the 12 selected standards were used in a combinatoric μXANES fitting only the spectra of 6 appeared to be present in the spectra of all the treatments.

The fractional contribution of each of the analysed standard compounds to the Zn spectrum of the root was assumed to be directly proportional to the fraction of Zn present in that form in the plant root (Terzano et al., 2008). The goodness of the fit was estimated by calculating the residual *R* factor of the fit; $\Sigma_i$ (experimental-fit) $^2$ / $\Sigma_i$ (experimental) $^2$, where the sums are over 103 data points as flattened μ (E). A lower *R* factor represents a better match between the fitted standard spectra and the sample spectrum (Terzano et al., 2008).

### 5.2.4. Statistical Analysis

All treatment means were tested for normal distribution using Anderson-Darling's normality test. All means are of equal variance and are normally distributed (Table

5.3). Analysis of variance followed by Tukey's HSD test ($p<0.05$) was used to identify significant differences in plant growth, percentage soil Zn removed, Zn yield in plant biomass, Zn bioaccumulation factor and Zn translocation factor between means of pots. Growth means of the two plants were calculated per pot before the mean from each of the 3 pots were subjected to Analysis of variance and mean separation. All statistical analyses were conducted using Minitab 16 software (Minitab$^{TM}$ Inc., USA).

Table 5.3(a). Results of Anderson Darling's (AD) normality test for means of week five plant height, above ground and below ground plant biomass. All treatment means are normally distributed (P-value > 0.05)

| **Treatments** | | **AD-value** | **P-value** |
|---|---|---|---|
| Week five plant height | B | 0.393 | 0.351 |
| | BZn | 0.263 | 0.321 |
| | BPZn | 0.312 | 0.424 |
| | BRZn | 0.298 | 0.463 |
| | BRPZn | 0.236 | 0.645 |
| Above ground biomass | Bo | 0.314 | 0.419 |
| | BZn | 0.254 | 0.578 |
| | BPZn | 0.219 | 0.711 |
| | BRZn | 0.305 | 0.445 |
| | BRPZn | 0.107 | 0.177 |
| Below ground biomass | Bo | 0.222 | 0.306 |
| | BZn | 0.357 | 0.364 |
| | BPZn | 0.246 | 0.606 |
| | BRZn | 0.241 | 0.295 |
| | BRPZn | 0.219 | 0.235 |

Table 5.3(b). Results of Anderson Darling's (AD) normality test for means soil Zn removed, fractional soil Zn contents, Zn yield in plant biomass, Zn translocation and bioaccumulation factors. All treatment means are normally distributed (P-value > 0.05)

| Treatments | | AD-Value | P-Value |
|---|---|---|---|
| Soil Zn removed (plant basis) | BZn | 0.329 | 0.416 |
| | BPZn | 0.192 | 0.619 |
| | BRZn | 0.182 | 0.62 |
| | BRPZn | 0.190 | 0.628 |
| Soil Zn removed (soil basis) | BZn | 0.289 | 0.302 |
| | BPZn | 0.279 | 0.328 |
| | BRZn | 0.190 | 0.629 |
| | BRPZn | 0.191 | 0.621 |
| Exchangeable soil Zn | BZn | 0.409 | 0.111 |
| | BPZn | 0.254 | 0.402 |
| | BRZn | 0.415 | 0.105 |
| | BRPZn | 0.399 | 0.118 |
| Reducible soil Zn | BZn | 0.220 | 0.514 |
| | BPZn | 0.352 | 0.178 |
| | BRZn | 0.207 | 0.556 |
| | BRPZn | 0.233 | 0.474 |
| Oxidiseable soil Zn | BZn | 0.205 | 0.562 |
| | BPZn | 0.296 | 0.285 |
| | BRZn | 0.421 | 0.510 |
| | BRPZn | 0.207 | 0.554 |
| Residual soil Zn | BZn | 0.191 | 0.625 |
| | BPZn | 0.246 | 0.428 |
| | BRZn | 0.342 | 0.194 |
| | BRPZn | 0.230 | 0.487 |
| Zn in above ground biomass | BZn | 0.290 | 0.531 |
| | BPZn | 0.389 | 0.601 |
| | BRZn | 0.151 | 0.331 |
| | BRPZn | 0.189 | 0.521 |
| Zn in below ground biomass | BZn | 0.277 | 0.333 |
| | BPZn | 0.189 | 0.531 |
| | BRZn | 0.381 | 0.415 |
| | BRPZn | 0.269 | 0.631 |
| Zn Translocation factor | BZn | 0.219 | 0.517 |
| | BPZn | 0.218 | 0.518 |
| | BRZn | 0.239 | 0.453 |
| | BRPZn | 0.126 | 0.392 |
| Zn Bioaccumulation factor | BZn | 0.217 | 0.522 |
| | BPZn | 0.222 | 0.510 |
| | BRZn | 0.236 | 0.464 |
| | BRPZn | 0.227 | 0.324 |

## 5.3. Results

### 5.3.1. Plant growth promotion by bacteria

The height of live plants was measured at 6 weeks after planting in soil contaminated with 400 mg $kg^{-1}$ of Zn (note that the results are similar to the one presented in Chapter 4 and are being summarised here as a reminder to the reader). The result shows that un-inoculated *B. juncea* plants (BZn) were highly susceptible to Zn contamination and they were shorter in height than the plants inoculated with bacteria (Figure 5.1a). Although plants inoculated with *P. brassicacearum* (BPZn) appeared to be taller than the BZn plants, plant heights in the two treatments were statistically the same. Plants inoculated with *R. leguminosarum* (BRZn) however grew significantly taller than un-inoculated plants and plants inoculated with *P. brassicacearum*. The tallest plants under Zn contamination were plants inoculated with the combination of the two bacterial strains (BRPZn) and they were significantly taller than the BZn, BPZn and BRZn plants. Furthermore, above ground and below ground dry weight of plants biomass that was harvested after 6 weeks was measured (5.1b).

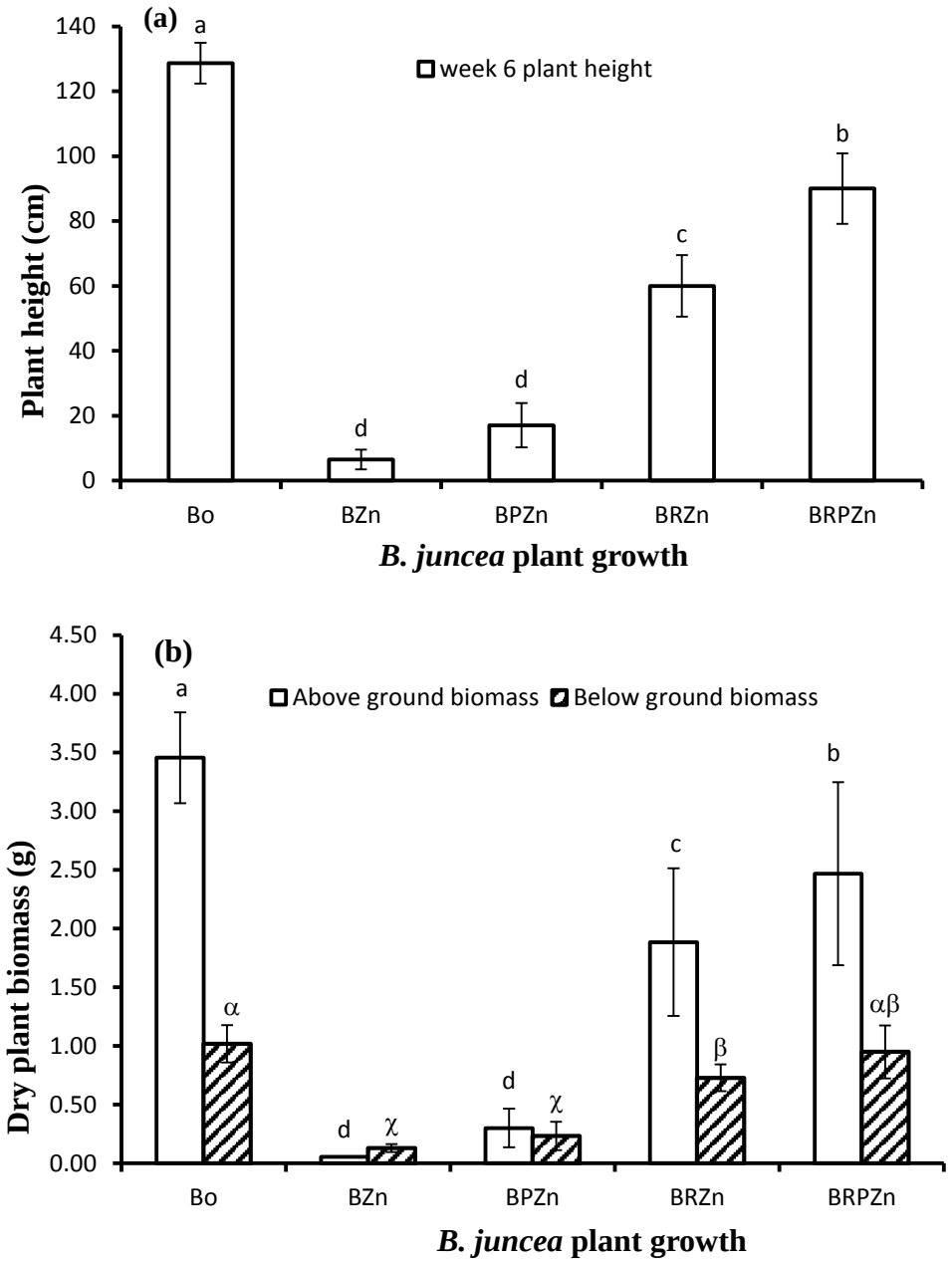



**Figure 5.1: (a) Plant height and (b) above and below ground dry plant biomass (b) in un-inoculated *B. juncea* in un-contaminated soil (Bo) and in soil contaminated with Zn (BZn) under single (BPZn, BRZn) and dual (BRPZn) bacterial inoculation with *R. leguminosarum* (R) and *P. brassicacearum* (P) at 6 weeks after planting. Figure shows that the best plant growth under Zn contamination is in plants inoculated with the two bacterial strains (BRPZn)**

As observed in plant height assessment, there were no statistically significant differences between the above and below ground plant dry biomass in un-inoculated plants and in plants inoculated with *P. brassicacearum* (5.1b). Below ground and above ground weights of dry plant biomass in plants inoculated with *R. leguminosarum* were however significantly more than the weights of biomass in the BZn and BPZn treatments. The highest amount of both above ground and below ground dry biomass under Zn contamination was in plants inoculated with the two bacterial strains (BRPZn).

It is worthy of note that the weight of below ground biomass in BRPZn is statistically the same with the weight of below ground biomass of plant in soil that was not contaminated with Zn, an indication that dual bacterial inoculation helps the plant root to overcome the deleterious effect of Zn contamination on growth. Soil Zn concentration and Zn bioaccumulation in the treatments were further analysed to assess if the enhanced plant growth leads to improved Zn accumulation and soil remediation.

### 5.3.2. Effect of bacteria on Zn remediation, bioaccumulation and translocation

Sequentially fractionated Zn contents in the soil samples were measured and Zn phytoextraction in the different treatments of the contaminated soil was compared by determining the percentage of Zn removed from the soil, Zn concentration in dry plant biomass and calculating Zn bioaccumulation and translocation factors.

By comparing the total concentration of accumulated Zn in plant biomass after the experiment with the level of Zn contamination at the beginning of the experiment, treatments under bacteria inoculations significantly recorded better Zn remediation

over the BZn treatment with a maximum Zn pytoextration efficiency of 25.8% observed under the BRPZn treatment (Figure 5 .2).

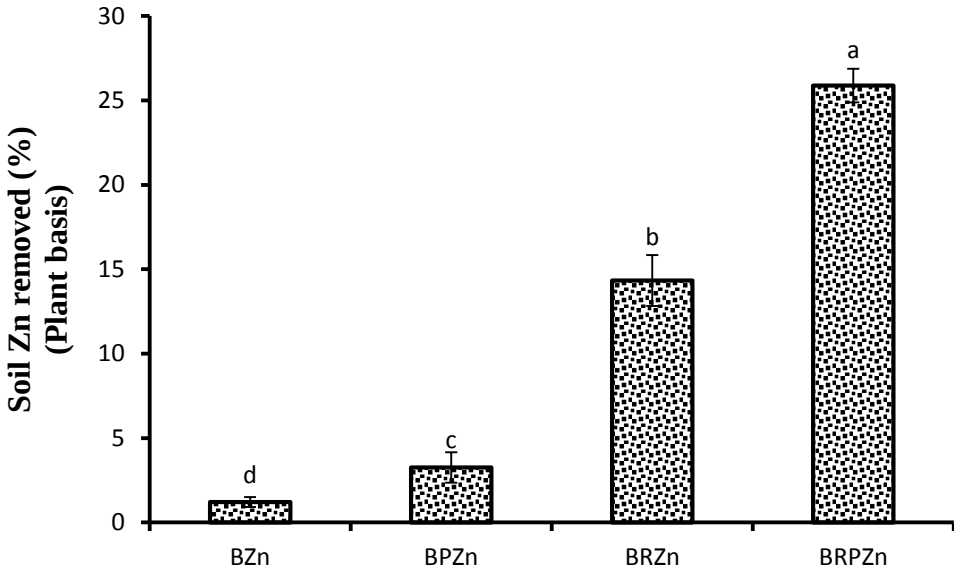



**Figure 5.2: Percentage of soil Zn removed in Zn contaminated soil under *B. juncea* un-inoculated (BZn) inoculated with *P. brassicacearum* (BPZn), *R. leguminosarum* (BRZn) and combinations of the two bacterial strains (BRPZn). Figure shows that inoculation of plants with *R. leguminosarum* and its combination with *P. brassicacearum* significantly enhanced soil Zn remediation compared to the use of *P. brassicacearum* (BPZn) and plant without bacterial inoculation (BZn)**

However, by comparing results of soil Zn contents in pots under phytoremediation with pots without phytoremediation, a much higher soil Zn removal values were observed in all the treatment with the highest Zn removal percentage (51.20 % ) in the BRPZn treatment (Fig.5.3).

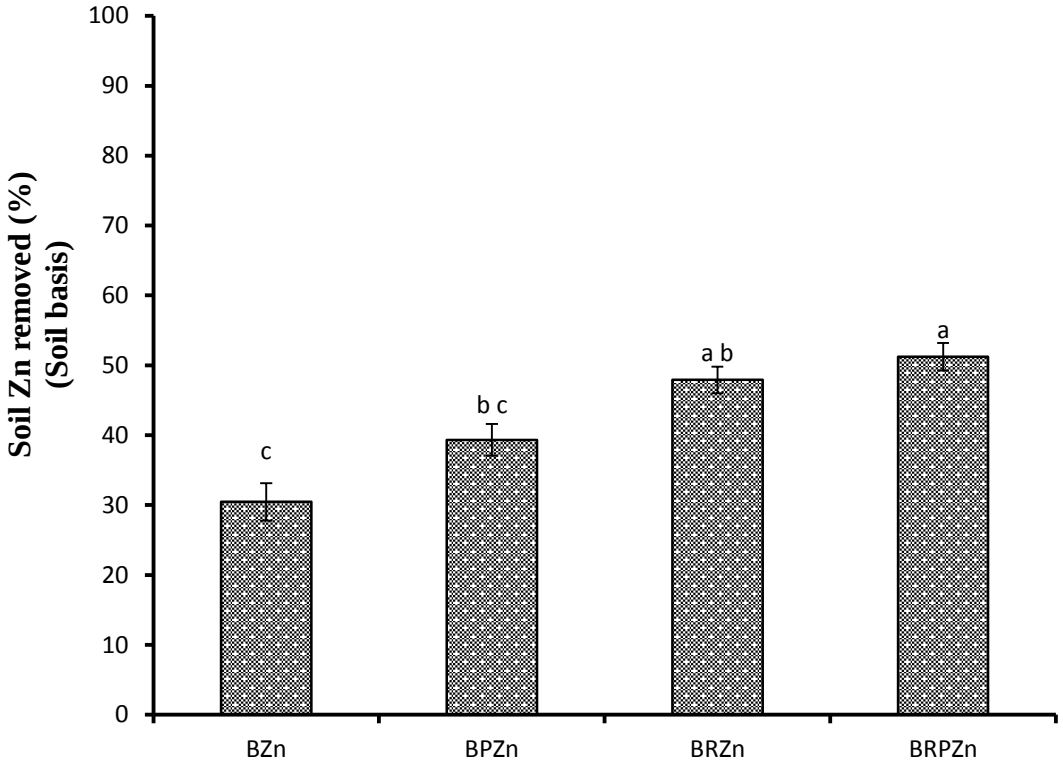



**Figure 5.3: Percentage of soil Zn removed in Zn contaminated soil under *B. juncea* un-inoculated (BZn) inoculated with *P. brassicacearum* (BPZn), *R. leguminosarum* (BRZn) and combinations of the two bacterial strains (BRPZn). Figure shows that inoculation of plants with *R. leguminosarum* and its combination with *P. brassicacearum* significantly enhanced soil Zn remediation compared to the use of plant without bacterial inoculation**

The difference between the plant and soil based Zn remediation results ranges from 25 – 35 % among the treatments (Figure 5.4).

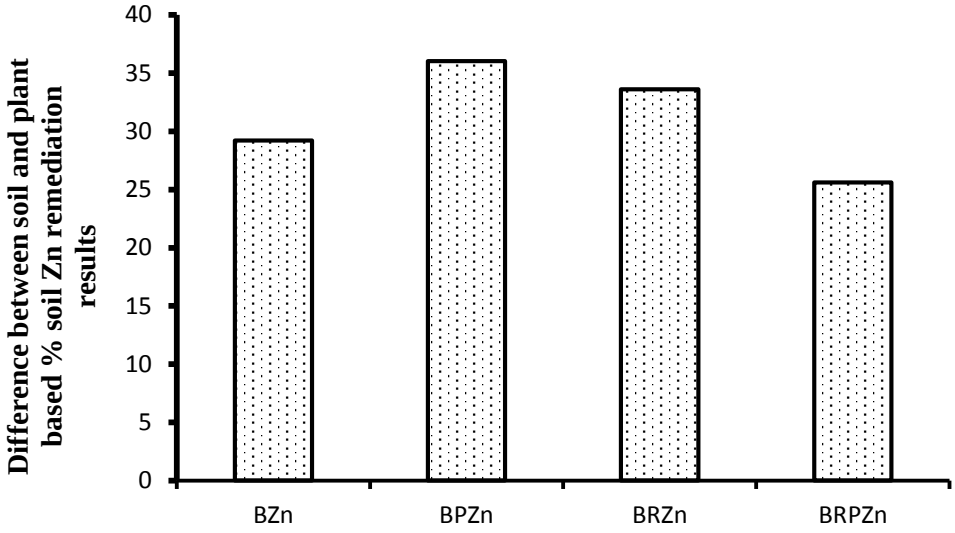



**Figure 5.4: Differences between soil and plant based percentage soil Zn remediation results soil under *B. juncea* un-inoculated (BZn) inoculated with *P. brassicacearum* (BPZn), *R. leguminosarum* (BRZn) and combinations of the two bacterial strains (BRPZn)**

This significant differences between the plant and soil based results may be traced to possible leaching of Zn from the pots during the watering process over the course of the experiment. Zn was added to the soil as Zn sulphate solution and existed predominantly in the water leachable (exchangeable) form in the soil (see Figure 5.5). This source of possible Zn loss might have reduced the amount of bioavailable Zn fraction for plant uptake over the course of the experiment. Moreover, the trays placed under the perforated pots were not washed back into the pots after the experiment before pots were sampled for analysis. It is likely that the some Zn residues might have deposited on the tray. Standard methods for establishing pot experiments and analysing samples from pot experiments were adopted in this study (Bennett et al., 2003, Hernández-Allica et al., 2008) and it was not clear that any leaching onto the saucer may have contributed to Zn loss and needed to be corrected for. However, control treatments (Zn contaminated pots without plants) were

established with the phytoremediation treatments and were watered exactly the same, for which the added Zn was recovered through the extraction process. Nevertheless, the results of Zn removal percentages consistently showed that *R. leguminosarum* (BRZn) and its combination with *P. brassicacearum* (BRPZn) significantly enhanced soil Zn removal when compared to the treatments without bacteria inoculation (BZn) (Figure 5.2 and 5.3).

The fractional Zn contents of the soil were further analysed in terms of their geochemical associated as defined operationally by the extraction scheme. The exchangeable (the fraction of Zn that is readily available) Zn in the soil under un-inoculated plants (BZn) was significantly higher than in soils under plants that were inoculated with bacteria (Figure 5.5).

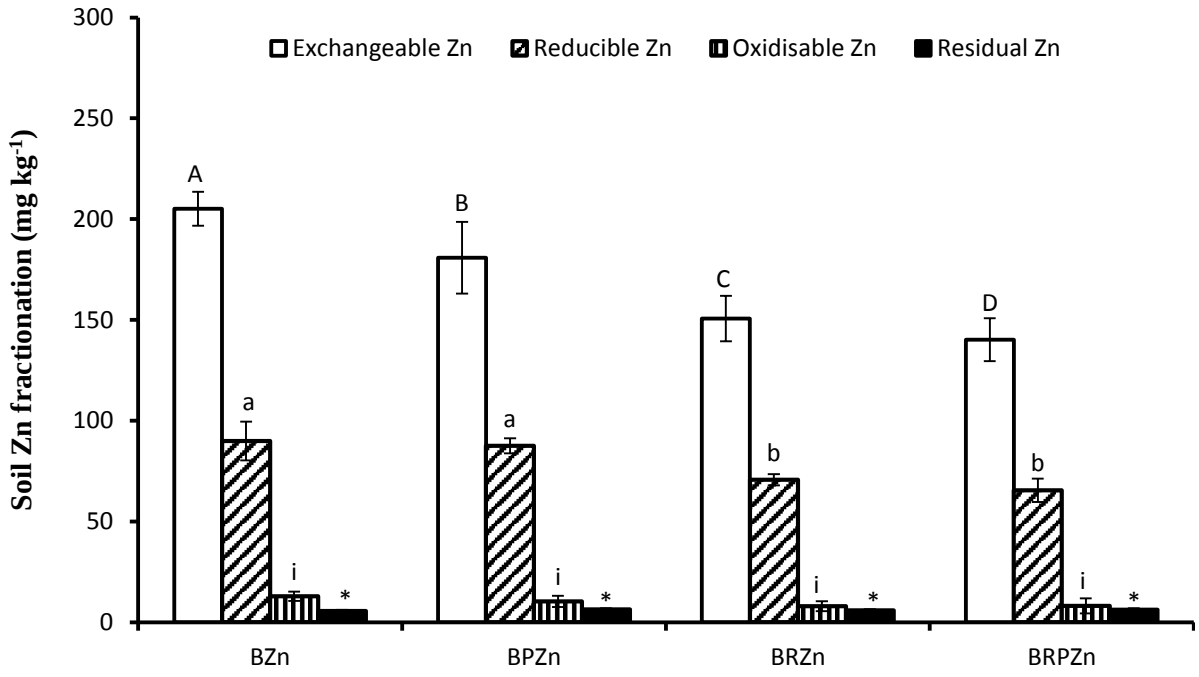



**Figure 5.5: Fractional Zn contents in remediated soil in un-inoculated plants (BZn) and plants inoculated with *R. leguminosarum* (BRZn), *P. brassicacearum* (BPZn) and bacteria combinations (B*RP*Zn) under Zn contamination for 6 weeks. Bars are means Zn contents from each of the experimental pots. Error bars show standard errors. Different letters indicate significant ($p<0.05$) differences in Zn contents. Figure shows that the best remediation of exchangeable Zn is in soil under plants inoculated with the two bacterial strains (BRPZn).**

The concentration of exchangeable Zn in soil under the plants inoculated with *R. leguminosarum* was significantly lower than in soil under the plants inoculated with *P. brassicacearum.* The lowest exchangeable soil Zn contents were however observed in soil under the plants that were inoculated with both *R. leguminosarum* and *P. brassicacearum* (BRPZn). Furthermore, reducible Zn contents in the soil of the BRZn and BRPZn treatments were significantly lower than in soil under BPZn and BZn treatments. The oxidisable and residual soil Zn contents were the same in all treatments.

Furthermore, Zn contents in above and below ground plant biomass were compared. The result of the analysis shows that un-inoculated plants have the lowest Zn contents in their above and below ground biomass (Figure 5.6).

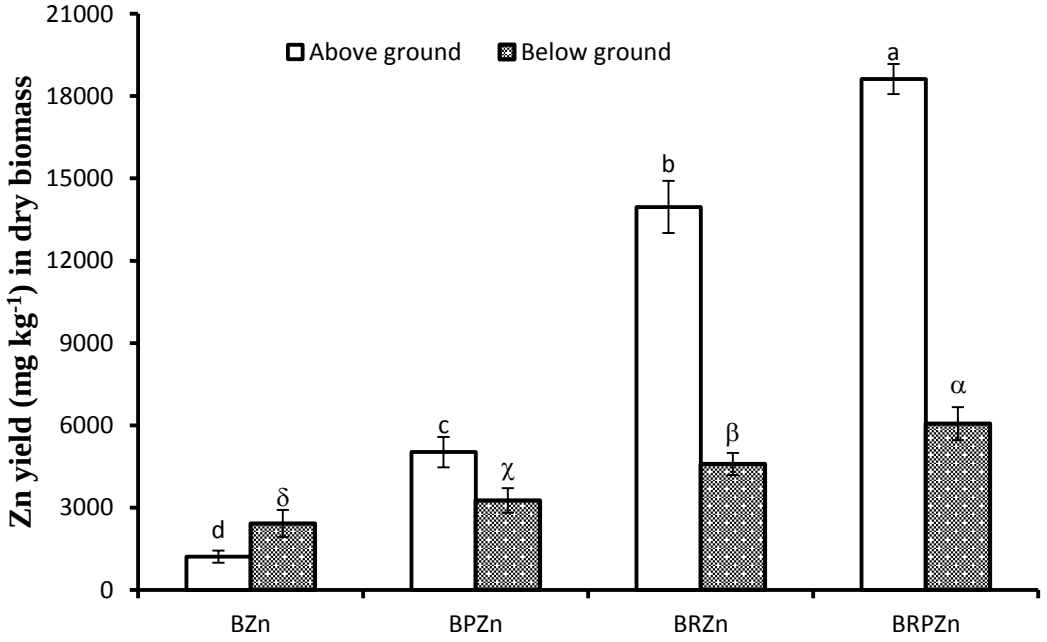



**Figure 5.6: Zn concentrations in above and below ground plant biomass of un-inoculated plants (BZn) and plants inoculated with *R. leguminosarum* (BRZn), *P. brassicacearum* (BPZn) and bacteria combinations (BRPZn) at 6 weeks after planting in Zn contaminated soil. Bars are means Zn contents from plants established in each of the experimental pots. Error bars show standard errors. Different letters/symbols indicate significant ($p<0.05$) differences in Zn contents. Figure shows that bacterial inoculations significantly enhance Zn accumulation in both the above and below ground plant biomass**

Zn yield in the biomass of plants inoculated with *P. brassicacearum* was significantly higher than in un-inoculated plants. Inoculation of plants with *R. leguminosarum* significantly enhanced Zn accumulation in both the above and below ground biomass relative to plants inoculated with *P. brassicacearum*. Most importantly, Zn contents in both above and below ground dry biomass of plants inoculated with the two bacteria (BRPZn) were significantly more than all the other treatments.

Furthermore, the BZn plants also have the lowest Zn bioaccumulation factor (BF) and translocation factor (TF) (Figure 5.7). Although the BPZn plants also showed increased values, significantly higher Zn bioaccumulation and translocation was observed in BRZn and BRPZn plants.

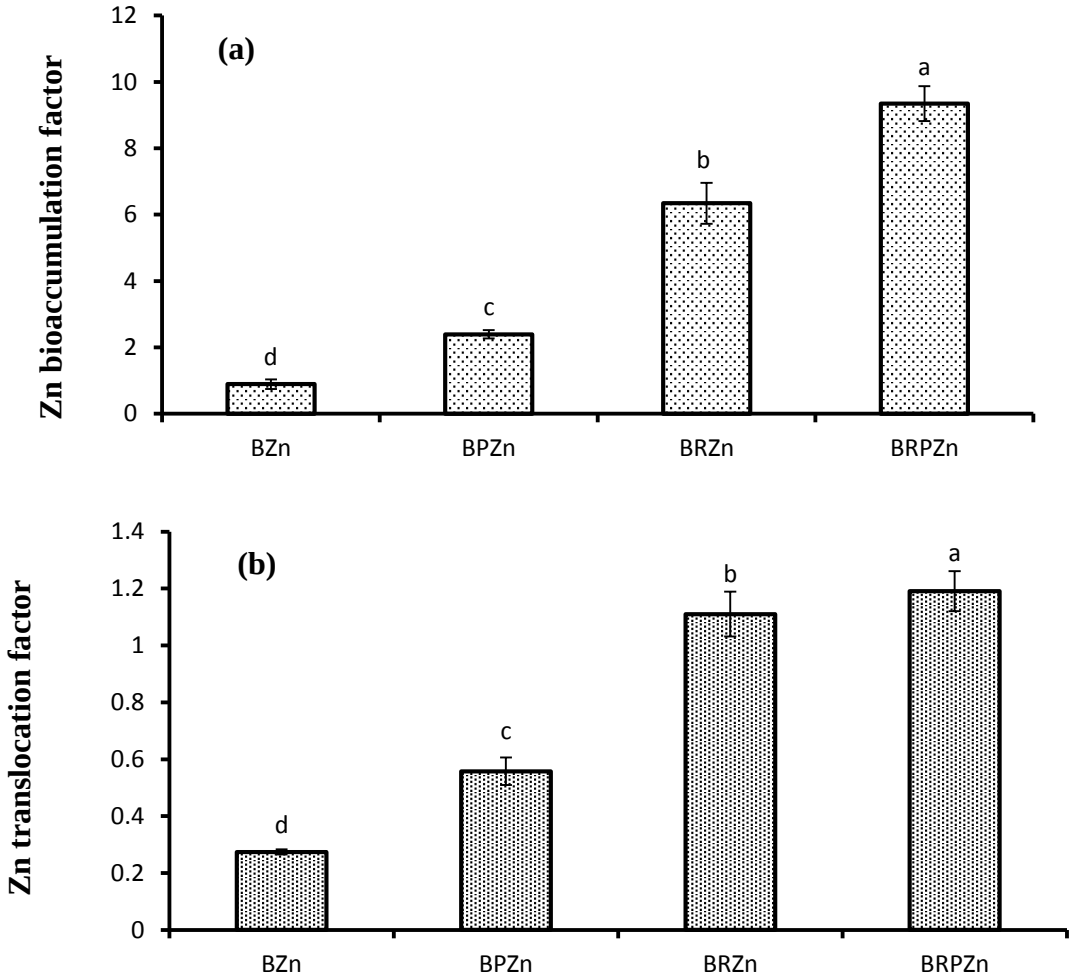



**Figure 5.7: Zn (a) bioaccumulation and (b) translocation factor in un-inoculated plants (BZn) and plants inoculated with *P. brassicacearum* (BPZn) and *R. leguminosarum* (BRZn), and bacteria combinations (BRPZn) at 6 weeks after planting in Zn contaminated soil. Bars are mean bioaccumulation and translocation factors for each pot. Error bars show standard errors. Different letters/symbols indicate significant ($p<0.05$) differences between treatments (n=3). Figure shows that inoculation of plants with bacteria enhances Zn bioaccumulation and root to shoot translocation of Zn**

In all the experiments, it is very clear that plants under bacteria inoculation accumulated significantly more Zn but, instead of the plants suffering retarded growth, they grew better. This paradox of better plant growth in the face of higher metal bioaccumulation was further investigated by examining Zn sequestration and speciation in the *B. juncea* plant roots using a combination of μXRF mapping and μXANES analysis.

### 5.3.3. Synchrotron based root μXRF imaging and XANES analyses

The μXRF mapping of Zn in fresh root biomass clearly shows that PGPB increased Zn bioaccumulation in roots (Figure 5.8).

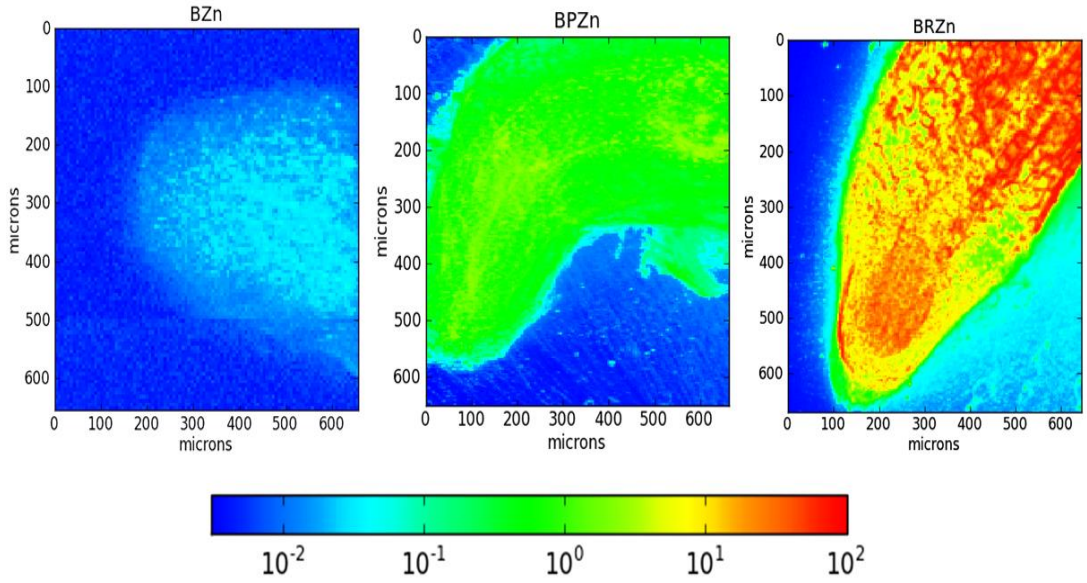



**Figure 5.8: Synchrotron based μXRF images of Zn distribution in BZn, BPZn and BRZn plant roots. Zn counts were normalized to incoming beam intensity and the beam detector was at the same distance from the sample for the acquisition of the maps. Colour bars ($log_{10}$ scale) indicate Zn counts in plant roots from highest (red) to lowest (blue). Figure shows higher Zn bioaccumulation of Zn in the roots of fresh plants under bacterial inoculation than in un-inoculated root.**

The maps confirm the findings of the dry root biomass analysis with the BRZn plant showing more Zn accumulation than BPZn and BZn plants. Moreover, Zn in BZn maps appears to be distributed more or less uniformly throughout the roots, whereas BPZn plants appear to accumulate slightly higher Zn concentrations (see the yellow patches at the middle of BPZn map) at the centre of the root than anywhere else. Moreover, a combination of dominant yellow and red patches with few visible green spots in BRZn maps suggests localized differences in Zn concentration within the plant root with the patterns conspicuously different from the BPZn and BZn maps.

It is also possible that the PGPB may have changed the biochemistry of Zn in the plant root, and this may be responsible for the better growth despite high Zn accumulation in the bacteria inoculated. Hence, μXANES spectra were acquired from high concentration Zn regions in the roots to determine Zn speciation. The Zn μXANES spectra of the root samples were compared with 12 selected standard compounds (Figure 5.9).

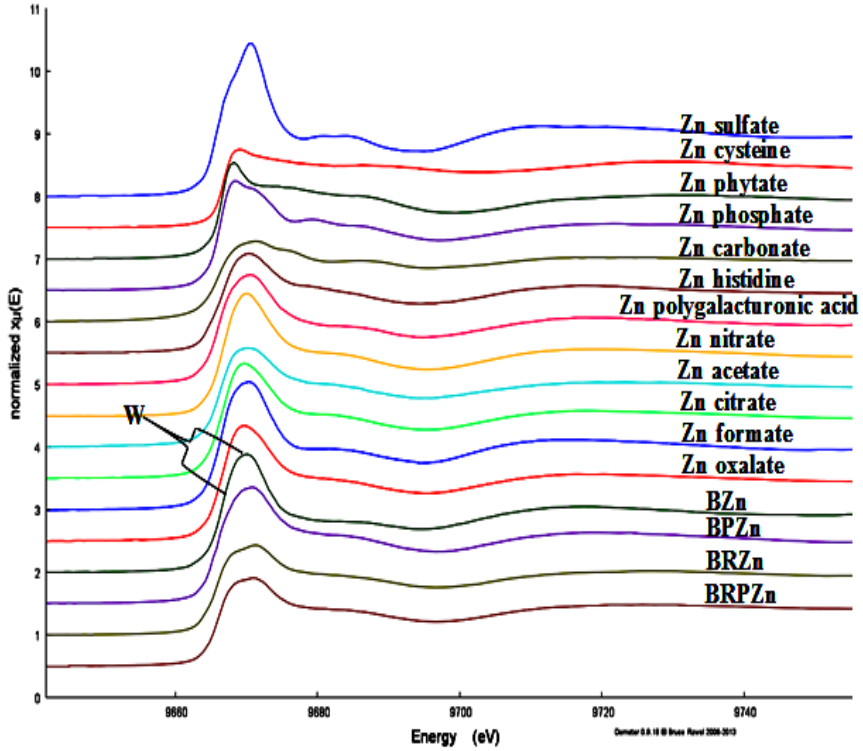



**Figure 5.9: Normalized XANES of selected Zn standard compounds and Zn in BZn BPZn, BRZn and BRPZn plant root. W shows the Zn K-edge white line in the BZn XANES**

A white line is a rising absorption edge that lead to a sharp intense peak, a position of which is very sensitive to oxidation state (Huggins et al., 2000). It is noticeable that the white line (see **W** in Figure 5.9) of the Zn K-edge μXANES in un-inoculated plant root (BZn) of 1.3 is much larger than those of BPZn, BRZn and BRPZn of 0.7,

0.5 and 0.45, respectively. Moreover, the μXANES spectra of BPZn are dissimilar to those of BRZn and BRPZn. LCF was used to determine the speciation of Zn in the root sample μXANES spectra (Figure 5.10).

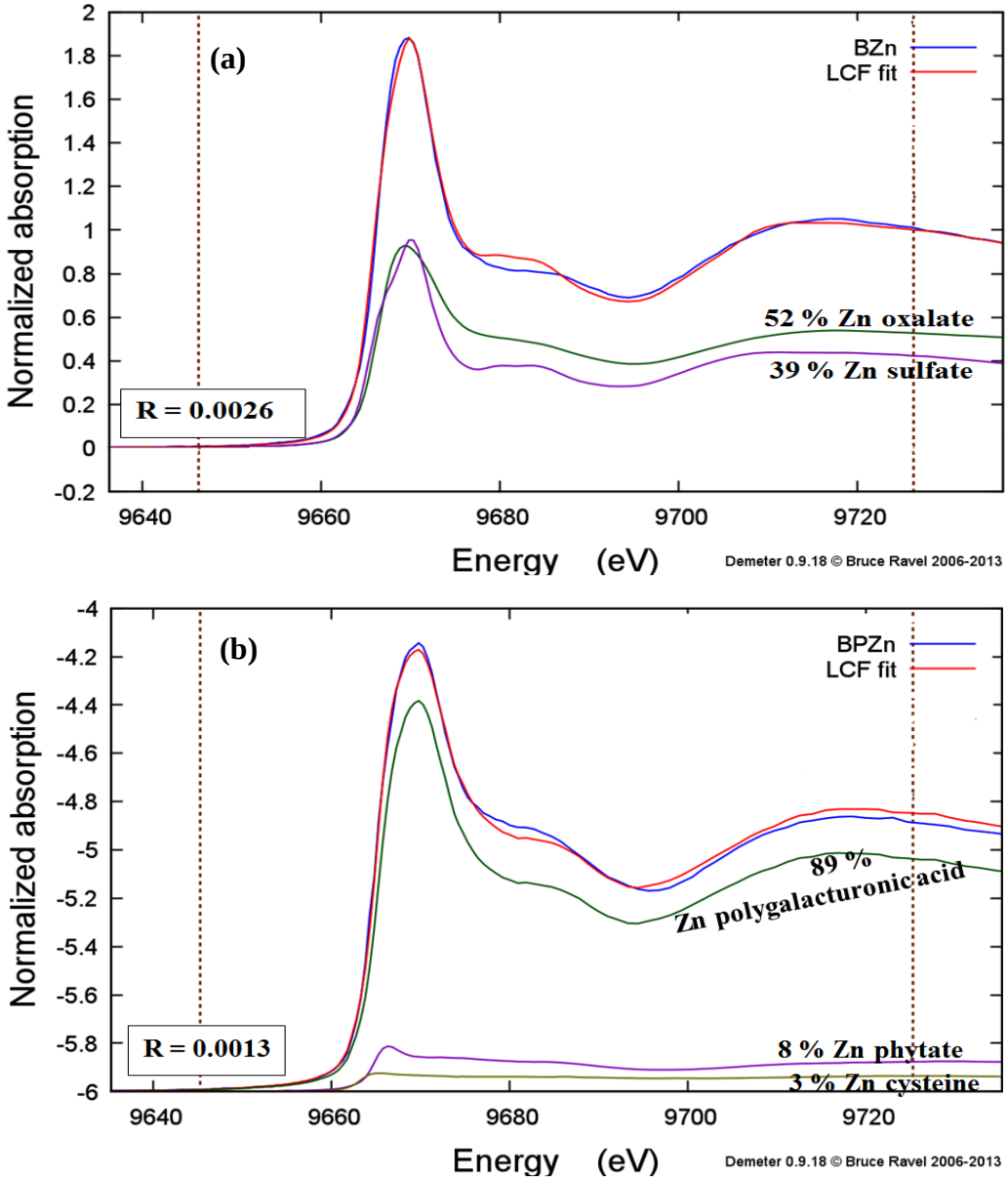
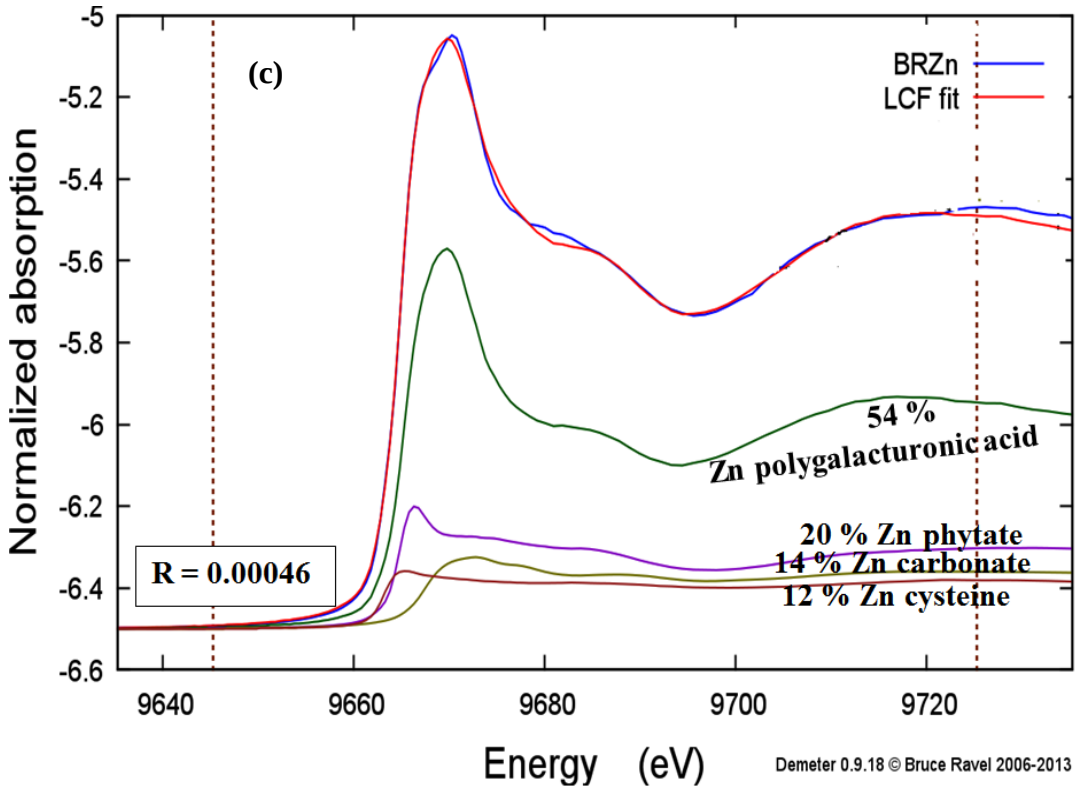



**Figure 5.10: K-edge XANES fitting, R factor and % Zn compound composition for (a) un-inoculated *B. juncea* plant root (BZn) and (b) *B. juncea* plant root inoculated with *P. brassicacearum* (BPZn). Figure shows that inoculation of plant with PGPB lead to changes in Zn speciation in plant root.**



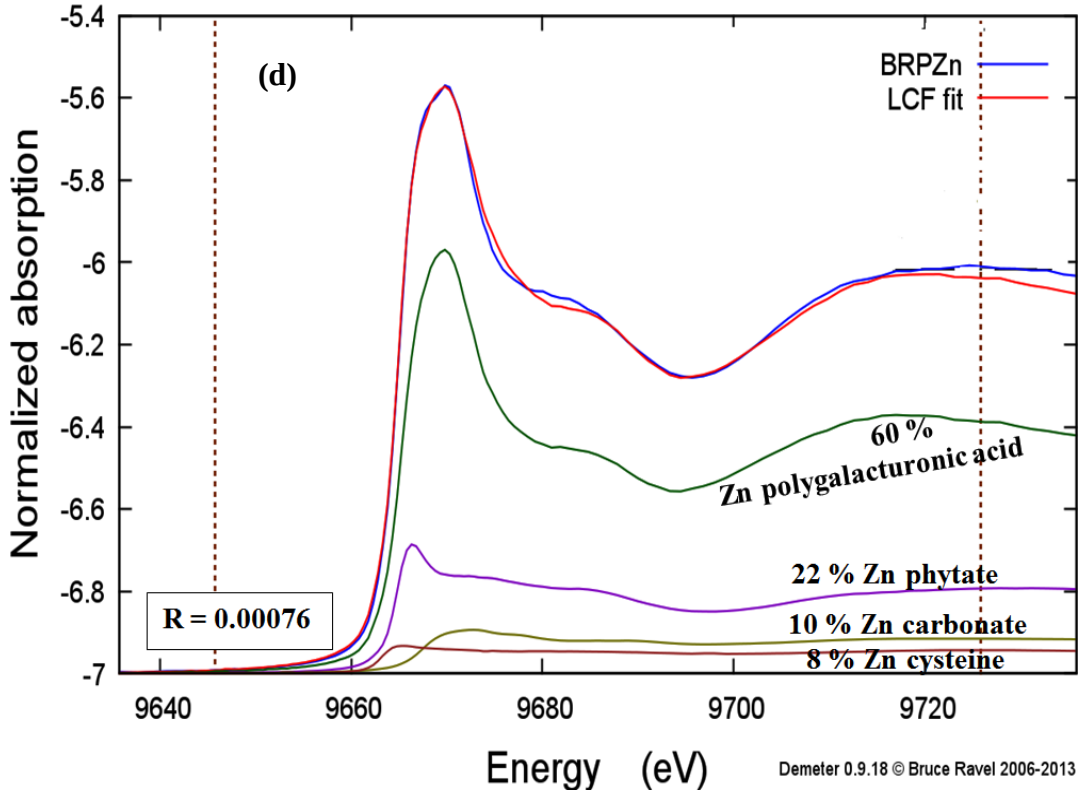




**Figure 5.10: K-edge XANES fitting, R factor and % Zn compound composition for (c) *B. juncea* plant root inoculated with *R. leguminosarum* (BRZn) and (d) combination of *P. brassicacearum and R. leguminosarum* (BRPZn). Figure shows that inoculation of plant with PGPB lead to changes in Zn speciation in plant root.**

The un-inoculated plant root (BZn) has the highest R value (Figure 5.10a) and the fit with all combinations of the standards was not as good as the inoculated root samples (Figure 5.10b-d), making it difficult to determine root Zn speciation with confidence. The best fit presented is consistent with Zn being in the form of Zn oxalate and Zn sulfate, but there is probably another component that was not available in the selected standard spectrum used for the LCF. The LCF fits for Zn in the roots of the inoculated plants are substantially better as shown by the lower R values (Figure 5.10b-d). They indicate that Zn is mainly stored as Zn polygalacturonic acid in the roots of BPZn, BRZn and BRPZn plants. The results also suggest a higher Zn phytate and Zn cysteine production capacity in plants inoculated with *R. leguminosarum* than in plants inoculated with *P. brassicacearum*. While Zn was not stored as Zn carbonate in the root of BPZn plants, a significant proportion of Zn existed as Zn carbonate in the roots of BRZn and BRPZn plants.

## 5.4. Discussion

The main findings of this study are:

(i) *R. leguminosarum* and its combination with *P. brassicacearum* significantly promoted the growth of *B. juncea* plants under Zn contamination.

(ii) Zn yield in plant biomass, Zn bioaccumulation and translocation factor, and phytoremediation of exchangeable soil Zn fraction were significantly enhanced in plants inoculated with *P. brassicacearum* (BPZn) than in un-inoculated plants (BZn) under Zn contamination.

(iii) Zn yield in plant biomass, percentage soil Zn removed, phytoremediation of both exchangeable and reducible Zn, Zn bioaccumulation and translocation factor were significantly higher in plants inoculated with *R. leguminosarum* (BRZn) and combinations of the two bacterial strains (BRPZn) than in BPZn and BZn treatments under Zn contamination.

(iv) μ-XRF imaging of fresh root samples confirms the results of dry biomass chemical analysis with higher Zn accumulation in plants inoculated with the PGPB than in un-inoculated plant.

(v) There were significant differences in Zn speciation between the root of plants inoculated with the PGPB and un-inoculated plant roots, with Zn significantly stored in the form of Zn cysteine and Zn phytate in the BRZn and BRPZn plants than in the BPZn plants.

Better plant growth in plants inoculated with PGPB compared to un-inoculated plants under metal contamination has been widely observed in many studies and the use of PGPB and metal remediating plants in a microbial-assisted phytoremediation system is gaining attention (Glick, 2003, Zhuang et al., 2007, Khan et al., 2009). In this study, *R. leguminosarum* significantly surpassed *P. brassicacearum* in promoting the growth of *B. juncea* under Zn contamination. *R. leguminosarum* bv. *trifolii* is renowned for its plant growth promoting ability in un-contaminated environments and it is amongst the most exploited species of root-nodule bacteria in agriculture worldwide due to its well-documented capacity for nitrogen fixation (Chen et al., 1991, Reeve et al., 2010). However, in Chapter 4 it was demonstrated that improvement in plant nutrition contributed little to plant growth promotion especially

due to use of fertile soil in this research, and it was suggested that the bacterial strain might have attenuated the toxicity of Zn in the plants by mechanisms unrelated to nutrient fixation.

Moreover, the results from soil Zn remediation and Zn accumulation in plants is of similar pattern to the results of the plant growth parameters, an indication that better plant growth leads to higher Zn accumulation in plants resulting to higher soil Zn remediation. This phenomenon has been observed in other studies and enhanced metal phytoremediation has been widely associated with healthier plant growth due to the plant growth promoting effects of bacteria on plants under metal contamination (Sheng and Xia, 2006, Li et al., 2007, Kuffner et al., 2008). Increase in root biomass (under bacteria inoculation) has been suggested to lead to increase in exchange and absorption of Zn ions into root biomass (Lasat et al., 1996). Increased solubilisation of unavailable forms of Zn-bearing compounds present in contaminated media through chelation, ligand-induced dissolution and organic acids secretion by plant growth promoting bacteria has also been reported to increase metal bioaccumulation in plants (Sessitsch et al., 2013). Solubilisation is likely to be an important mechanism mainly in cases where the metal exists in non-bioavailable (e.g. adsorbed or co/precipitated) forms, hence the fact that that are also decrease in the reducible Zn fraction (more strongly adsorbed to iron and manganese oxides) suggests that solubilisation is a small but likely factor in the phytoremediation results in this study.

Furthermore, bioaccumulation factor (BF) and translocation factor (TF) are important parameters for assessing the ability of metal accumulating plants to absorb metals from contaminated soil and the subsequent translocation of the absorbed metal from the root to harvestable aerial biomass. For effective toxic metal phytoremediation, BF and TF should be greater than 1.0 (Wei and Chen, 2006). Although inoculation of plants significantly increased the BF and TF of *B. juncea* plants, effective Zn phytoremediation was only achieved in plants inoculated with *R. leguminosarum* (BRZn) and its combination with *P. brassicacearum* (BRPZn). Moreover, the remediation of more available and reducible soil Zn fractions in BRZn and BRPZn makes inoculation of *B. juncea* with *R. leguminosarum* and its combination with *P. brassicacearum* more ideal in situations where effective phytoextraction of Zn is the desired goal (Bhargava et al., 2012).

However, despite the stunted growth in plants inoculated with *P. brassicacearum,* percentage soil Zn removed in the treatment was statistically the same with percentage soil Zn removed in plants inoculated with *R. leguminosarum*. Although Zn bioaccumulation factor was significantly lesser in BPZn plants than in BRZn plants and translocation factor in BPZn plants was less than 1.0, inoculation of *B. juncea* with *P. brassicacearum* significantly enhanced Zn bioaccumulation factor from 0.89 (in un-inoculated plants) to 2.39. Therefore, *P. brassicacearum* significantly promoted Zn accumulation at the below ground plant component compared to un-inoculated *B. juncea* plants (BZn), and the bacterial strain can be used to promote the phytostabilisation of Zn by *B. juncea* in situations where phytostabilisation is the desired goal (Mendez and Maier, 2008).

Moreover, results from μXRF mapping of fresh root sample strongly support the results of dry roots chemical analysis with more Zn accumulation in the roots of plants inoculated with PGPB. Apart from the conspicuous increase in Zn bioaccumulation there are also conspicuous differences between the pattern of Zn accumulation in BPZn plants and BRZn plants. In plants without root nodules (like *B. juncea*), *R. leguminosarum* mainly resides at the rhizosphere of the plant roots (Schloter et al., 1997). *P. brassicacearum* on the other hand, was isolated from the root of a Brassica plant and has been shown to be capable of colonising interior root areas (Long et al., 2008). Although Zn appeared to be significantly accumulated at the outer spheres of the root in plants inoculated with the rhizospheric *R. leguminosarum*, with slightly higher Zn deposition in the interior of plant root inoculated with the endophytic *P. brassicacearum,* it has not yet been demonstrated whether the nature of bacterial colonisation of root corresponds with the pattern of Zn accumulation in plant root and if this has any influence on root growth under Zn contamination.

Nevertheless, it is likely that apart from increased Zn accumulation under bacteria inoculation, the PGPB induced different mode of Zn sequestration in the plant roots based on their nature of root colonisation. Organelles in the plant root have been observed to have different levels of tolerance to metal toxicity (Clemens, 2001, Hall, 2002). The vacuole of plant roots for example, have been reported to exhibit higher level of metal tolerance and sequestration (Clemens, 2006, Verbruggen et al., 2009). Cell walls and plasma membranes have also been observed to be capable of binding

significant amounts of metal ions to the root epidermis (Hall, 2002, Dalvi and Bhalerao, 2013). Preventing metal sensitive cellular components from having contacts with toxic concentration of metals, which is achieved by the ability of the metal tolerant components to sequester toxic concentrations, has therefore been suggested as metal tolerance mechanisms in plants under metal contamination (Clemens, 2001, Hall, 2002, Dalvi and Bhalerao, 2013). The better tolerance observed in BRZn may therefore be attributed to bacteria induced sequestration of toxic concentration of Zn at specialized cell components (probably at the cell wall) of the inoculated plant root.

However, the conspicuous changes in Zn speciation observed among the plant root of the studied treatments sufficiently explain the paradox of better growth in the face of higher Zn bioaccumulation in the root of plants inoculated with PGPB. Despite the higher R value in the LCF fits of the BZn plants compared to the other treatments, Zn was observed to be predominantly stored in the form Zn oxalate (~52%) and Zn sulphate (~39%) in the plant roots. Support for this partial interpretation is provided by other studies that showed Zn oxalate accumulation in the root of Zn-resistant ecotype *Silene cucubalus* and *Rumex acetosa* planted on Zn spiked nutrient medium (Mathys, 1977). The reaction of Zn with oxalate has been observed to form the stable Zn oxalate complex ($ZnC_2O_4$) with a stability constant, log $K$ = 4.68 (Sillén and Martell, 1964). High concentrations of this soluble acidic Zn oxalate complex in metal resistant plants have been linked with enhanced Zn toxicity to plants (Mathys, 1977). The Zn source in this experiment was inorganic $ZnSO_4.7H_2O$ so identifying a significant proportion of Zn in the plant root as $ZnSO_4$ is not unexpected.

Thus there is probably an association between the possible predominance of Zn as oxalate and sulphate in the root of *B. juncea* plants and the retarded growth of these plants in this study.

Furthermore, presence of Zn polygalacturonic acid in the inoculated roots and not in the un-inoculated roots suggests that it is induced by the inoculated PGPB. Polygalacturonic acid ($C_{12}H_{14}O_{12}Zn.4H_2O$, also known as pectic acid) within the cell wall (pectin) of plants has been shown to reduce metal toxicity to plants through the formation of metal-organic acid complexes (Cataldo et al., 2012, Dalvi and Bhalerao, 2013) via its carboxylic group (Deiana et al., 1980). The toxicity is mediated by immobilization of the Zn, binding the metal to root cell walls and thus restricting the interaction of the toxic metal with vital plant tissues (Manara, 2012). This factor may therefore be responsible for the significantly lower Zn translocation observed in BPZn plants than in the BRZn plants which had significantly lower form of Zn as Zn polygalacturonic acid in its root. Moreover, increasing organic acid secretion in plants has been recognised as a stress tolerance response mechanism, however it has also been shown to cause disruption of plant metabolism (Dalvi and Bhalerao, 2013). The lower plant growth and especially stunted root development in BPZn plants compared to the other inoculated plants may therefore be due to the occurrence of Zn predominantly as polygalacturonic acid in this treatment.

Moreover, it is likely that in plants inoculated with *R. leguminosarum* and in combination with *P. brassicacearum* this toxic effect is mediated by storing Zn in other forms, such as Zn phytate (8% in BPZn, 20% in BRZn, 22% in BRPZn) and Zn

carbonate (14% in BRZn and 10% in BRPZn).The results suggest a higher Zn phytate production capacity in *R. leguminosarum* than in *P. brassicacearum*. Formation of Zn phytate complexes in plant roots is increasingly being recognised as the main survival mechanism in plants under metal toxicity (Van Steveninck et al., 1990, Kopittke et al., 2011). Phytate has strong negatively charged phosphate groups that form very stable complexes with Zn (Crea et al., 2008, Kopittke et al., 2011). Significant accumulation of Zn phytate in the roots of non-hyperaccumulating *Arabidopsis lyrata*, *Deschampsia caepitosa* and *Eruca vesicaria* and hyperaccumulating *Brassica napus* and *Noccaea caerulescens* is thought to play a major role in regulating Zn toxicity (Terzano et al., 2008, Kopittke et al., 2011). Due to its high molecular size, great stability, insolubility and resistance to enzymatic hydrolysis, the endogenous Zn phytate complex may reduce Zn toxicity by immobilizing Zn to specialized cells of the endodermis and mediating the translocation of excessive Zn across plasma membranes of root cells, thus regulating Zn movement into the xylem and shoot (Van Steveninck et al., 1994, Terzano et al., 2008).

Results from LCF analysis suggest a higher proportion of Zn occurring as cysteine in BRZn plant roots (12%) compared to BPZn (3%). Phytochelatin, a cysteine-rich oligopeptide, has been reported to chelate metals in plants (Dişbudak et al., 2002, Dalvi and Bhalerao, 2013). Cytoplasmic complexation of Zn with cysteine isolates excessive Zn from metal sensitive enzymes and facilitates the translocation and accumulation of Zn in the vacuoles where the Zn cysteine complex becomes more resistant to protolytic degradation (Dalvi and Bhalerao, 2013).

Most gram-negative bacteria, like the *rhizobium* species used in this study, have been reported to possess cysteine containing glutathionine which has been suggested to be involved in metal ion binding and membrane transport (Crockard et al., 2002). This may also be responsible for the better Zn translocation from the root to the shoot of plants inoculated with *R. leguminosarum* observed in this study. It is also likely that the formation of Zn cysteine in plants inoculated with *R. leguminosarum* reduces Zn toxicity to the plant, thereby contributing to the better plant growth.

### 5.5. Conclusion and wider environmental implication of results

As hypothesised, *P. brassicacearum* and *R. leguminosarum* induced different levels of Zn toxicity attenuation through different modes of Zn sequestration and the formation of different types of Zn chelates in bacterial inoculated plant roots under Zn contamination. Moreover, bacteria induced Zn chelation with the phytochelatins and metallothioneins in the plant root appears to be a major mechanism through which bacteria promote plant growth under Zn contamination. It can also be concluded that better plant growth under bacterial inoculation will lead to higher Zn bioaccumulation and better soil Zn phytoremediation in Zn contaminated soil.

Inoculation of hyperaccumulating plants with bacteria that are genetically modified to improve their ability to secrete phytochelatins, and the use of transgenic plants are active areas of phytoremediation research (Bañuelos et al., 2006, Zhang et al., 2013b). This Chapter, however, suggests a microbial-phytoremediation system that combines the use of two or more bacteria on a hyperaccumulating plant, as a more affordable and sustainable method for remediation of soils contaminated with toxic metals.

Nevertheless more studies are required with different bacteria combinations and other toxic metals to confirm this assertion.

In this study, *R. leguminosarum* significantly promoted plant growth and Zn phytoremediation. Most leguminous plants harbour *R. leguminosarum* and other PGPB in their root. Chapter 6 will explore the possibility of a Zn tolerant leguminous plant conferring its inherence Zn tolerance to the *B. juncea* and enhancing Zn phytoremediation in a mixed planting system by combining the use of simple agronomic techniques with advanced synchrotron based X-ray Absorption Spectroscopy, as the basis for the analysis.

# 6. Mixed planting with a leguminous plant outperforms bacteria in promoting growth of a metal phytoremediator

This Chapter is based on the following manuscript currently under review in the Environmental and Experimental Botany Journal:

**Gbotemi A. Adediran**, Bryne T. Ngwenya, J. Frederick W. Mosselmans, Kate V. Heal, Barbra A. Harvie. Mixed planting with a leguminous plant outperforms bacteria in promoting growth of a metal phytoremediator.
As the lead author, I performed the experiments and was involved in laboratory analysis. Data analysis and preparation of the first draft of the manuscript was carried out by me. The co-authors provided support and guidance on the scope and design of the study and also specialist laboratory support on XAS analysis.

## 6.1. Introduction

Heavy metals are poisonous metallic elements and metalloids associated with environmental contamination and biological toxicity (Alkorta et al., 2004). Although released into the environment through some geogenic processes, much of the environmental contamination by heavy metals originates from anthropogenic activities, including industrial production, energy production, mining and agricultural activities (Zereini et al., 2005, Hu and Cheng, 2013). The large inventory of heavy metal contaminated sites (Lado et al., 2008) and the cross media environmental contamination (Gopalakrishnan et al., 2008) and persistence of metals have necessitated the development of chemical and mechanical remediation methods (Lothenbach et al., 1997, Montinaro et al., 2007). These methods are effective, however they are also environmentally destructive, expensive and unsustainable (Mulligan et al., 2001a).

By contrast, plants are natural miners of nutrients and other elements from the environment (Sheoran et al., 2009). The unique ability of plants to remediate toxic metals (metal phytoremediation) has been actively researched, leading to

identification of metal hyperaccumulators, plants that accumulate metals in their tissue (Vara Prasad and de Oliveira Freitas, 2003). Unfortunately, hyperaccumulators are not necessarily tolerant and may be subject to metal toxicity at high metal contaminations, leading to poor remediation efficiency (Kumar et al., 1995, Ebbs and Kochian, 1997).

Meanwhile, some plants that do not necessarily have metal remediating abilities but are capable of growing in metal-contaminated environments with little or no sign of stress have also been identified (Kováčik et al., 2006, Broadley et al., 2007). Studies suggest that the tolerance to high soil metal contamination in these hypertolerant plants may be linked to the production of metallothioneins and phytochelatines that act as metal chelators within the rhizosphere of the plants, leading to reduced metal bio-toxicity (Kinnersley, 1993, Blindauer, 2008, Fischer et al., 2014).

Inoculating phytoremediators with plant growth promoting bacteria (PGPB) has been demonstrated to promote growth and metal remediation in contaminated environments (Burd et al., 2000, Ma et al., 2011a) and in Chapter 5 of this research, metal chelation with metallothioneins and phytochelatines (phytate and cysteine) was identified as the possible mechanism behind enhanced plant growth and metal bioaccumulation in plants inoculated with PGPB. However, the effects of PGPB on plant growth promotion under high soil metal contamination are not always satisfactory and remediation efficiency is still relatively low (Zhuang et al., 2007).

A mixed planting system that involves a target crop and a leguminous plant is widely accepted as a cropping system to enhance the growth of the target crop under nutrient

deficiency (Malézieux et al., 2009, Qiao et al., 2012). However, to my knowledge, the possibility of a non-remediating but metal-tolerant leguminous plant conferring its resistance to a plant with remediating ability in a mixed planting system under metal contamination has not been considered previously.

It was therefore hypothesised that:

(i) Co-planting a phytoremediator with a Zn hypertolerant plant would confer the Zn-tolerance benefits of the latter to the phytoremediator, leading to improved growth and phytoremediation.

(ii) Legume-phytoremediator mixed planting system will outperform the use of PGPB in promoting plant growth and Zn phytoremediation under Zn contamination.

(iii) The difference in Zn tolerance between the hypertolerant plant and the Zn phytoremediator will be due to differences in the nature of Zn species synthesised in the roots of the plants.

This Chapter presents results of an experiment in which *Brassica juncea*, an established phytoremediator but with poor tolerance to high concentrations of the heavy metal zinc (Zn), was co-planted under Zn contamination with *Vicia sativa*, a leguminous plant with no known phytoremediation activity but established to be hypertolerant to Zn in preliminary studies. The effect of the mixed planting system was also compared with the use of plant growth promoting *Pseudomonas brassicacearum* and *Rhizobium leguminosarum* bacteria to enhance phytoremediation.

## 6.2. Experimental

### 6.2.1. Materials

The experiment used the phytoremediating plant *B. juncea* L. *Czern* (Indian mustard) and the leguminous plant *Vicia sativa* subsp. *sativa* L. (cultivated vetch). The plant growth promoting bacteria used were *Pseudomonas brassicacearum* subsp**.** *brassicacearum* and *Rhizobium leguminosarum* bv. *trifolii*. A standard soil for pot experiments, Scotts Levingston F2+S Seed & Modular growth medium (Green-tech Ltd., UK),(Vicente et al., 2012) was used (see Chapter 3 for detailed description). The soil was sterilised and dried in an autoclave at the start of the experiment. The background soil Zn content was 48±10 mg $kg^{-1}$ (mean ± standard error of n=3 analyses). The soil was spiked with Zn using Zn sulphate solution. Sulfate was chosen rather than nitrate to avoid the confounding effects of nitrate as a macronutrient.

### 6.2.2. Plant growth and toxicity tolerance assessment

A pot experiment was conducted in a glasshouse with 0.5 kg of soil placed in 5 L plastic pots located in a saucer for each experimental replicate. Treatments that required Zn contamination were spiked with Zn sulphate solution at the rate of 400 mg Zn $kg^{-1}$ soil dry weight, a concentration typical of contaminated agricultural soils in the UK (Baker et al., 1994b). The same volume of deionised water was added to the controls. Pots were then watered to field capacity and allowed to stand in the glasshouse for a week before seed planting. Seeds were surface sterilised, washed in sterile deionised water and dried under aseptic conditions (Kumar et al., 2008). Five seeds were planted per pot. (See detailed description of seed washing and

sterilisation methods in Chapter 3). Seedlings were weeded to two plants per pot 5 d after emergence. Each treatment was replicated in 3 pots which were randomly distributed in the glasshouse space. To prevent water stress the pots were kept moist throughout the experiment with deionised water. Plant height above the soil surface was measured weekly for 5 weeks after seed planting when plants under Zn contamination were still alive and those in uncontaminated treatments were almost 1 meter in height.

### 6.2.3. Mixed planting and bacterial inoculation

The ability of the leguminous *V. sativa* plant to promote the growth of *B. juncea* in a mixed planting system was evaluated and compared to the use of PGPB under the same experimental conditions as described above. (See detailed description of experimental conditions at the glasshouse in Chapter 3). For the mixed planting treatment, one *B. juncea* plant and one *V. sativa* plant were established at a spacing distance of 1.5 cm in the same 5 L pots. For the bacteria inoculated treatments, cells of *P. brassicacearum* and *R. leguminosarum* were cultured in nutrient broth at 30°C to an exponential growth stage, batched and washed three times in sterile 1 M sodium perchlorate and sterile deionised water. Surface sterilised seeds of *B. juncea* plants were incubated under aseptic conditions at 30°C in a suspension of bacteria in sterile water (absorbance of 0.5 at 600 nm) for 3 hours. Seeds for the control treatments were soaked in sterile deionised water in the same conditions. For treatments that required bacterial inoculation as well as mixed planting with *V. sativa*, inoculated *B. juncea* seeds were planted at a spacing distance of 1.5 cm to the *V.*

*sativa* plant in the same 5 L pot. A full description of all experimental treatments is presented in Table 6.1.

Table 6.1. A description of all experimental treatments

| Treatment | Description |
|---|---|
| Bo, Vo | Sole planted *B. juncea* and *V. sativa* plants in soil not contaminated by Zn |
| -Zn | Zinc contaminated soils without plants |
| BZn, VZn | Sole planted *B. juncea* and *V. sativa* plants under Zn contamination |
| BPZn | Sole planted *B. juncea* plants inoculated with *P. brassicacearum* under Zn contamination |
| BRZn | Sole planted *B. juncea* plants inoculated with *R. leguminosarum* under Zn contamination |
| BVZn | *B. juncea* co-planted with *V. sativa* under Zn contamination |
| BPVZn | *B. juncea* inoculated with *P. brassicacearum* and co-planted with *V. sativa* under Zn contamination |
| BRVZn | *B. juncea* inoculated with *R. leguminosarum* and co-planted with *V. sativa* under Zn contamination |

### 6.2.4. Evaluation of phytoremediation efficiency

The soil in each of the three replicates per treatment was sampled at the end of the 5 week growing period and two 1 g oven-dried sub-samples were extracted into exchangeable, reducible and oxidisable Zn soil fractions using a modified BCR sequential extraction method (Rauret et al., 1999) followed by residual soil Zn extraction with aqua regia (Rauret et al., 1999). Briefly, acetic acid (0.11 M $L^{-1}$), hydroxylamine hydrochloride (0.5 M $L^{-1}$), hydrogen peroxide (8.8 mL $L^{-1}$) and ammonium acetate (1 M $L^{-1}$) were used to obtain exchangeable, reducible and oxidisable Zn soil fractions respectively (Rauret et al., 1999). Residual Zn content was extracted in aqua regia, a 1:3 mixture of nitric and hydrochloric acids, for 3 hours at 110°C. Extracts were analyzed using inductively coupled plasma-optical emission spectroscopy (ICP-OES) using a Perkin Elmer Optima 5300 DV.

Extraction blanks were also analysed and subtracted from analytical results. Total Zn content was calculated as the sum of the concentration in the four fractions. Phytoremediation efficiency was estimated by subtracting total soil Zn content in pots under phytoremediation from contaminated pots without plants.

### 6.2.5. Analysis of Zn speciation in the plant roots

Live plants were transported to the Diamond Light Source, Didcot, UK, at 5 weeks after planting. Plant roots were selected for Zn distribution and speciation analysis on the microfocus spectroscopy beamline I18 (Mosselmans et al., 2009). Roots were chosen because they are the first plant organ to have contact with Zn, roots of other plants and soil bacteria, and the site where the first reaction to metal toxicity and sequestration takes place (Zhou et al., 2013). Synchrotron micro X-ray fluorescence (μXRF) maps of Zn in the root samples were collected at cryogenic temperature of -80°C in fluorescence mode using a nine-element germanium solid state detector. The collected μXRF data were processed into images using PyMCA 4.4.1(Solé et al., 2007).

μXANES data were collected from Zn hot spots identified in the element maps, along with 12 Zn model compounds (Zn oxalate, phosphate, histidine, cysteine, phytate, polygalacturonate, formate, sulfate, nitrate, citrate, acetate and carbonate) potentially involved in Zn speciation within the plant-soil system studied (Terzano et al., 2008, Kopittke et al., 2011). Consecutive spectra from the same point were examined for possible beam damage. For each of the treatments, 6 good μXANES spectra were selected, merged and subjected to further analysis. μXANES data from

the root samples were compared to the model compounds using a least-squares algorithm involving Linear Combination Fitting (LCF) in Demeter 0.9.18.

The goodness of the fit was estimated by calculating the residual *R* factor of the fit, $\Sigma_i$ (experimental-fit)$^2$ / $\Sigma_i$ (experimental)$^2$, where the sums are over 103 data points as flattened mu (E). A lower *R* factor represents a better match between the fitted standard spectra and the sample spectrum (Terzano et al., 2008).

### 6.2.6. Statistical analysis

The mean weekly plant heights above the soil surface (of the two plants per pot) and the Zn concentrations of the two soil sub-samples from each pot 5 weeks after planting were used for further data analysis. All treatment means were tested for normal distribution using Anderson-Darling's normality test. All means are of equal variance and are normally distributed (Table 6.2). Significant ($p<0.05$) differences between the treatments, each comprising three replicate pots, were identified by applying either two-sample t-tests or 1-way Analysis of Variance followed by Tukey's multiple comparison tests. All statistical analyses were conducted using Minitab 16 software (Minitab$^{TM}$ Inc., USA).

Table 6.2(a). Results of Anderson Darling's (AD) normality test for means of weekly plant heights. All treatment means are normally distributed (P-value > 0.05)

| Treatments | | AD-value | P-value |
|---|---|---|---|
| Week 1 plant height | Bo | 0.189 | 0.631 |
| | BZn | 0.277 | 0.334 |
| | Vo | 0.189 | 0.631 |
| | VZn | 0.230 | 0.487 |
| | BPZn | 0.277 | 0.334 |
| | BRZn | 0.358 | 0.169 |
| | BVZn | 0.277 | 0.334 |
| | BPVZn | 0.223 | 0.487 |
| | BRVZn | 0.313 | 0.487 |
| Week 2 plant height | Bo | 0.189 | 0.631 |
| | BZn | 0.23 | 0.487 |
| | Vo | 0.349 | 0.183 |
| | VZn | 0.23 | 0.487 |
| | BPZn | 0.194 | 0.609 |
| | BRZn | 0.192 | 0.62 |
| | BVZn | 0.255 | 0.399 |
| | BPVZn | 0.230 | 0.487 |
| | BRVZn | 0.241 | 0.487 |
| Week 3 plant height | Bo | 0.247 | 0.426 |
| | BZn | 0.230 | 0.487 |
| | Vo | 0.204 | 0.565 |
| | VZn | 0.330 | 0.214 |
| | BPZn | 0.204 | 0.565 |
| | BRZn | 0.192 | 0.620 |
| | BVZn | 0.249 | 0.312 |
| | BPVZn | 0.194 | 0.609 |
| | BRVZn | 0.249 | 0.312 |
| Week 4 plant height | Bo | 0.199 | 0.588 |
| | BZn | 0.204 | 0.565 |
| | Vo | 0.277 | 0.334 |
| | VZn | 0.319 | 0.235 |
| | BPZn | 0.193 | 0.614 |
| | BRZn | 0.249 | 0.312 |
| | BVZn | 0.20 | 0.338 |
| | BPVZn | 0.20 | 0.338 |
| | BRVZn | 0.23 | 0.487 |
| Week 5 plant height | Bo | 0.192 | 0.62 |
| | BZn | 0.189 | 0.631 |
| | Vo | 0.209 | 0.547 |
| | VZn | 0.206 | 0.557 |
| | BPZn | 0.183 | 0.349 |
| | BRZn | 0.255 | 0.399 |
| | BVZn | 0.191 | 0.623 |
| | BPVZn | 0.220 | 0.514 |
| | BRVZn | 0.240 | 0.449 |

Table 6.2(b). Results of Anderson Darling's (AD) normality test for means of soil Zn removed and fractional soil Zn contents. All treatment means are normally distributed (P-value > 0.05)

| Treatments | | AD-value | P-value |
|---|---|---|---|
| Soil Zn removed | BZn | 0.289 | 0.302 |
| | VZn | 0.223 | 0.507 |
| | BPZn | 0.279 | 0.328 |
| | BRZn | 0.19 | 0.629 |
| | BVZn | 0.207 | 0.556 |
| | BPVZn | 0.239 | 0.310 |
| | BRVZn | 0.243 | 0.307 |
| Exchangeable Zn | BZn | 0.409 | 0.111 |
| | VZn | 0.219 | 0.327 |
| | BPZn | 0.254 | 0.402 |
| | BRZn | 0.415 | 0.105 |
| | BVZn | 0.196 | 0.6 |
| | BPVZn | 0.199 | 0.587 |
| | BRVZn | 0.193 | 0.614 |
| Reducible Zn | BZn | 0.22 | 0.514 |
| | VZn | 0.101 | 0.274 |
| | BPZn | 0.352 | 0.178 |
| | BRZn | 0.207 | 0.556 |
| | BVZn | 0.223 | 0.507 |
| | BPVZn | 0.19 | 0.344 |
| | BRVZn | 0.287 | 0.307 |
| oxidiseable Zn | BZn | 0.205 | 0.562 |
| | VZn | 0.201 | 0.274 |
| | BPZn | 0.296 | 0.285 |
| | BRZn | 0.421 | 0.510 |
| | BVZn | 0.249 | 0.312 |
| | BPVZn | 0.230 | 0.487 |
| | BRVZn | 0.189 | 0.631 |
| Residual Zn | BZn | 0.191 | 0.625 |
| | VZn | 0.204 | 0.565 |
| | BPZn | 0.246 | 0.428 |
| | BRZn | 0.342 | 0.194 |
| | BVZn | 0.220 | 0.516 |
| | BPVZn | 0.242 | 0.4443 |
| | BRVZn | 0.190 | 0.630 |

## 6.3. Results and discussion

### 6.3.1 Plant growth and phytoremediation in the absence of bacterial inoculation and mixed planting

A qualitative assessment of plant growth in un-contaminated and Zn contaminated soil shows that the *B. juncea* (BZn) plants were significantly stunted while the *V. sativa* (VZn) plants exhibited tolerance to Zn contamination (Figure 6.1).

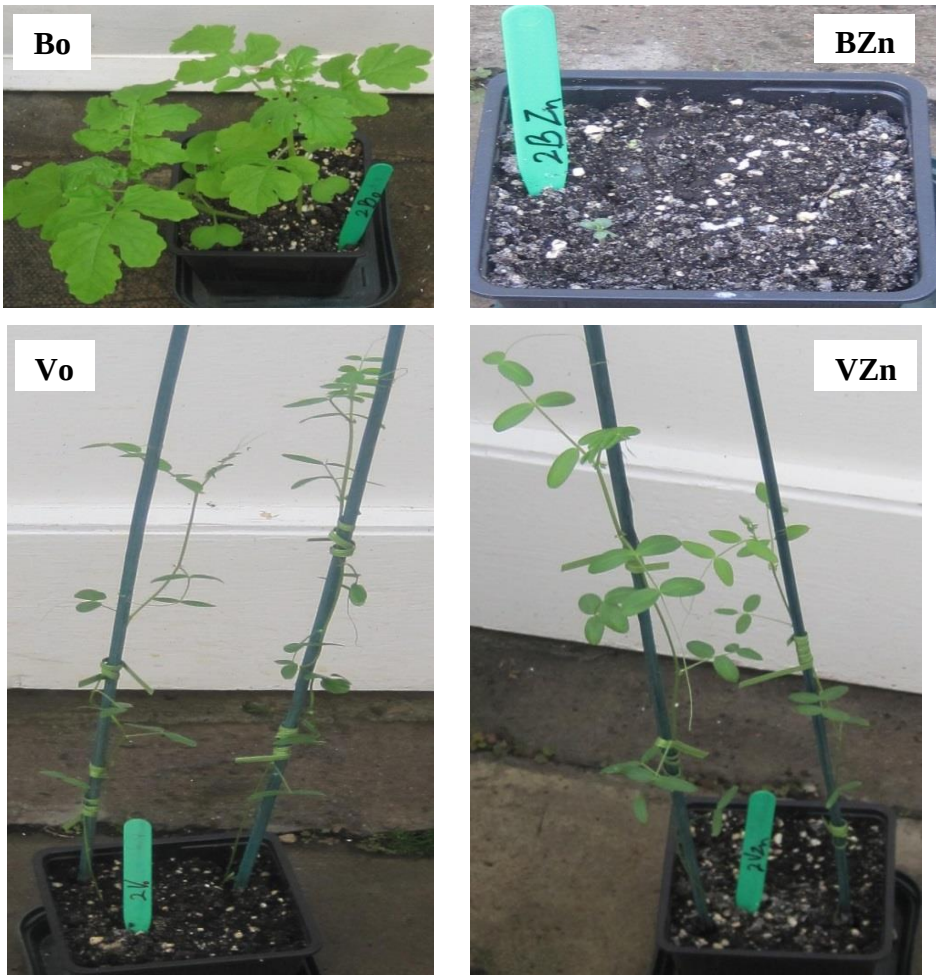



**Figure 6.1: *B. juncea* and *V. sativa* in uncontaminated soil (Bo and Vo) and in Zn contaminated soil (BZn and VZn) at 33 days after planting. Figure shows stunted growth in *B. juncea* under Zn contamination while the growth of *V. sativa* was not hindered under Zn contamination**

Moreover, the above ground growth of *B. juncea* and *V. sativa* plants in un-contaminated (Bo and Vo) and Zn contaminated (BZn and VZn) was evaluated weekly and compared over a period of 5 weeks (Figure 6.2).

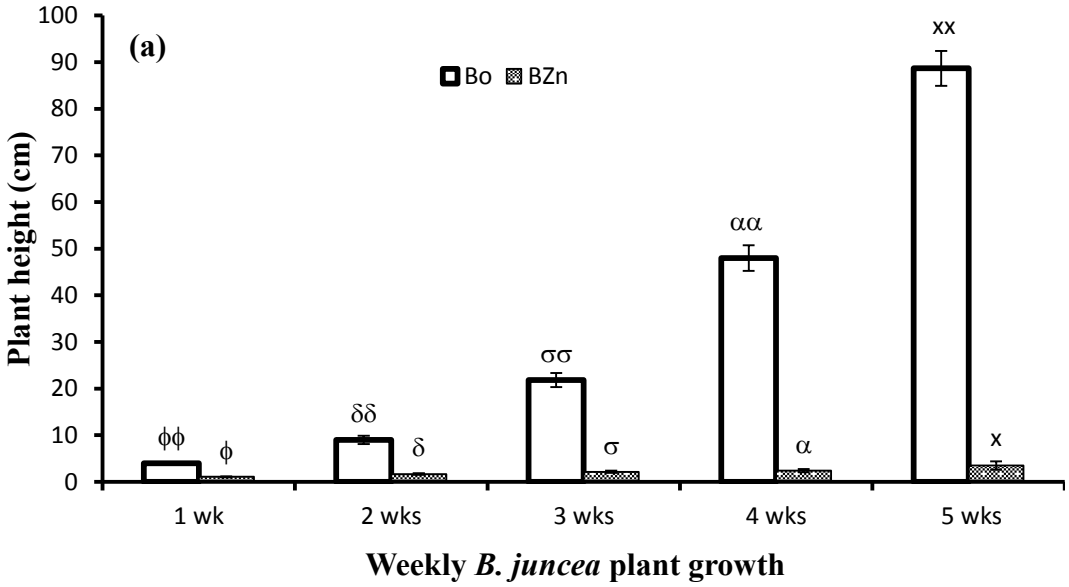




**Figure 6.2: Weekly plant height of *B. juncea* (a) and *V. sativa* (b) in uncontaminated soil (Bo and Vo) and in Zn contaminated soil (BZn and VZn). Bars are mean plant heights from the 3 experimental pots and error bars show standard errors. Weekly means were subjected to t-test pairwise comparison. Different symbols indicate significant ($p<0.05$) differences in weekly plant height between treatments (n=3). Figure shows that while the growth of *B. juncea* under Zn contamination is significantly stunted compared to the same plant in uncontaminated soil, there is no significant differences between the growth of *V. sativa* in Zn contaminated and uncontaminated soil at 5 weeks after planting.**

The *B. juncea* plants under Zn contamination compared to the *B. juncea* plants in soil not contaminated with Zn suffered significant stunted growth from week 1 to week 5 (Figure 6.2). Although the growth of the *V. sativa* plants under Zn (VZn) compared to the growth of *V. sativa* in soil not contaminated with Zn (Vo) were hindered for the first two weeks, there were no significant differences between Vo and VZn plants from week 3 to the week 5). The result shows that *V. sativa* is tolerant to Zn additions to soil at 400 mg $kg^{-1}$ whereas *B. juncea* is susceptible. Despite the vulnerability of BZn plants to this level of Zn contamination, they however have a significantly higher Zn remediation efficiency than the tolerant VZn plants (Figure 6.3).

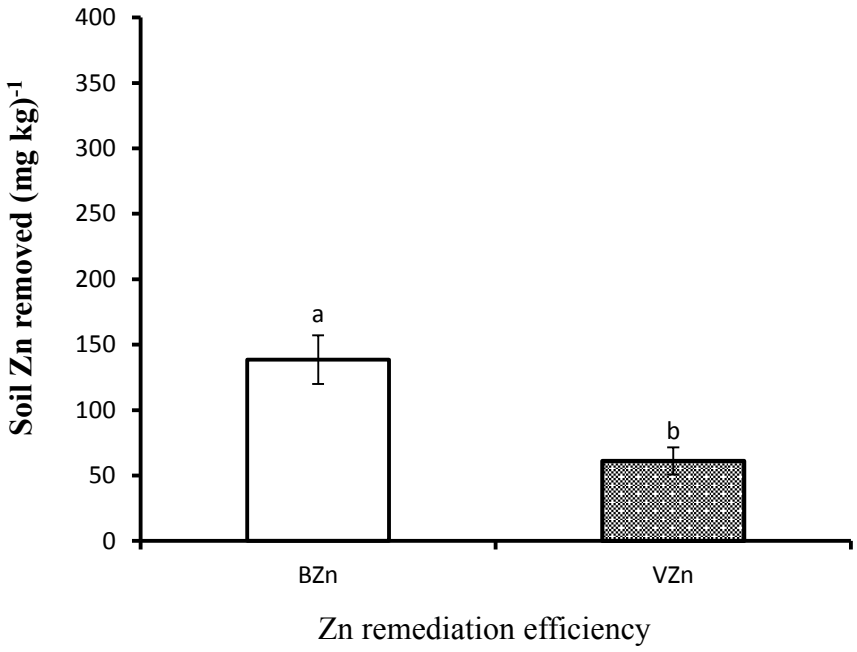



**Figure 6.3: Zn removed per mass of soil showing in soil under *B. juncea* (BZn) and *V. sativa* (VZn). Bars are mean values at 5 weeks after planting and error bars show standard errors (n=3). Different letters indicate significant ($p<0.05$) differences between treatments. Figure shows *B. juncea* as a better phytoremediator of Zn.**

Apart from higher Zn remediation under *B. juncea* plants, fractional analysis of Zn in soils under the two plants after 5 weeks shows that the *B. juncea* plants significantly remediate more exchangeable (the readily available) Zn fraction than the *V. sativa* plants (Figure 6.4).

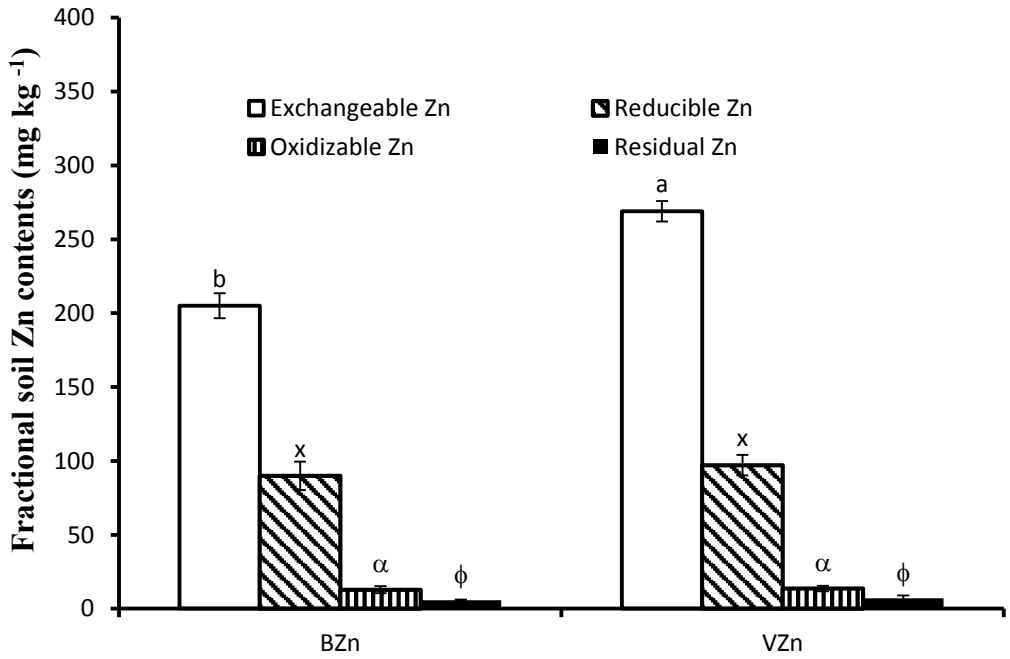



**Figure 6.4: Zn concentrations in different fractions of remediated soil under BZn and VZn. Bars are mean values at 5 weeks after planting and error bars show standard errors (n=3). Different letters/symbols indicate significant ($p<0.05$) differences between treatments. Figure shows *B. juncea* as a better phytoremediator of exchangeable Zn fraction**

The observed phytoremediation ability of *B. juncea* is well known (Ebbs and Kochian, 1998, Quartacci et al., 2006) but the Zn hyper-tolerance of *V. sativa* reported in this study is new. The *V. sativa* plants achieved their full growth potential under Zn contamination in contrast to the *B. juncea* plants. Enhancing the growth of *B. juncea* in Zn contaminated soils should therefore lead to a higher Zn phytoremediation. This is commonly achieved by inoculating phytoremediators with

PGPB (Glick, 2003, Zhuang et al., 2007, Khan et al., 2009); hence the use of PGPB to promote the growth and phytoremediation efficiency of *B. juncea* was evaluated.

### 6.3.2. Growth and Zn remediation efficiency of *B. juncea* under bacterial inoculation

At 5 weeks after planting in Zn contaminated soils, *B. juncea* plants inoculated with *R. leguminosarum* (BRZn) exhibited significantly higher plant growth than plants inoculated with *P. brassicacearum* (BPZn) which in turn had significantly higher growth than un-inoculated (BZn) plants (Figure 6.5a). Apart from the positive effect of bacteria inoculation on plant growth, the BPZn plants removed more soil Zn and reduced exchangeable soil Zn more than the BZn plants. The best Zn remediation under bacterial inoculation was however observed in the BRZn treatment, which also removed significantly more reducible soil Zn compared to the BZn and BPZn plants (Figures 6.5b and c).

Plant growth promotion by bacteria has been well studied, although the specific mechanisms through which bacteria promote plant growth under heavy metal contamination are still debatable. Mechanisms suggested to date include improved fixation, release and utilization of essential nutrients; and change in metal speciation to a less toxic form (Glick, 2005, Khan et al., 2009). In previous studies (Chapter 4 and Chapter 5), these possibilities has been evaluated and change in Zn speciation in the roots of *B. juncea* exposed to Zn has been suggested as the main mechanism of plant growth promotion by PGPB under Zn contamination.

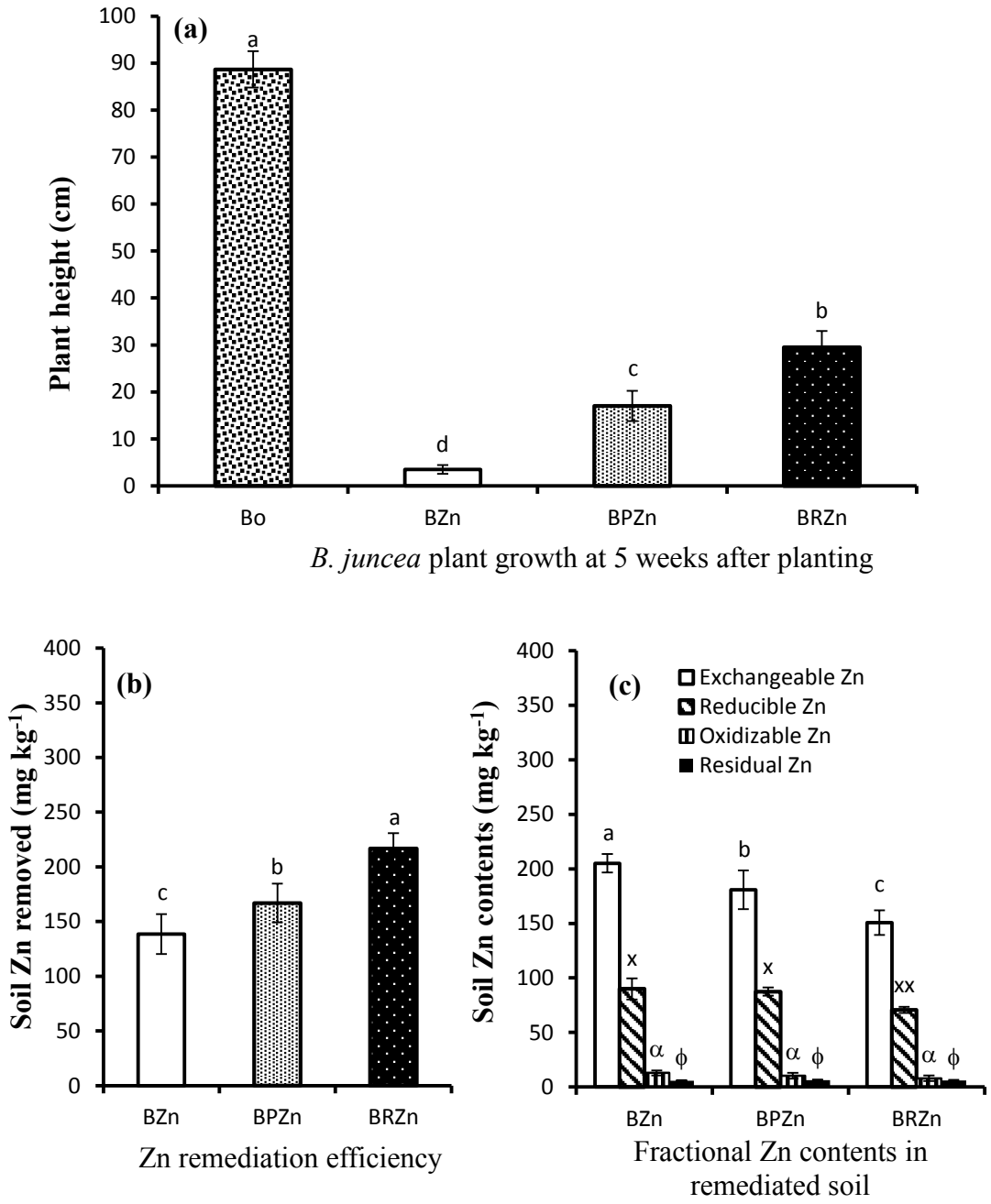



**Figure 6.5: (a) *B. juncea* plant height in uncontaminated soil (Bo) and under Zn contamination without bacteria inoculation (BZn), and inoculation with *P. brassicacearum* (BPZn) and *R. leguminosarum* (BRZn), (b) Zn removed per mass of soil and (c) Zn concentrations in different fractions of remediated soil under BZn, BPZn and BRZn plants. Bars are mean values at 5 weeks after planting and error bars show standard errors (n=3). Different letters/symbols indicate significant ($p<0.05$) differences between treatments. Figure shows that plant growth under Zn contamination and Zn phytoremediation improves with bacteria inoculation**

In Chapter 5, it was shown that likely mechanisms by which *R. leguminosarum* and *P. brassicacearum* ameliorate Zn toxicity to *B. juncea* were the formation of Zn complexes with polygalacturonic acid, phytate and cysteine. Although inoculation with PGPB promoted plant growth and resulted in improved phytoremediation, the growth of inoculated *B. juncea* was still significantly retarded, with a mean plant height of just 29 cm under BRZn compared to 89 cm for *B. juncea* growing in uncontaminated soil. A strategy that promotes the growth of phytoremediators under toxic soil metal concentrations to attain the level of growth in uncontaminated soil is therefore desirable for effective phytoremediation.

The use of leguminous plants to promote yield in a legume-crop mixed planting system is a well-known agronomic practice in nutrient deficient soils (Ghosh et al., 2007, Malézieux et al., 2009). The leguminous plant *V. sativa,* already demonstrated to be tolerant to Zn contamination (Figure 6.1-6.2) and which has also been reported to be associated with diverse species of bacteria (Lei et al., 2008) was chosen, and its ability to promote the growth and phytoremediation efficiency of *B. juncea* under Zn contamination was evaluated.

### 6.3.3. Growth and Zn phytoremediation efficiency of *B. juncea* co-planted with *V. sativa*

At 5 weeks after planting, *B. juncea* co-planted with *V. sativa* (BVZn) was significantly taller than sole planted *B. juncea* (BZn) upon exposure to Zn contamination (Figure 6.6a).

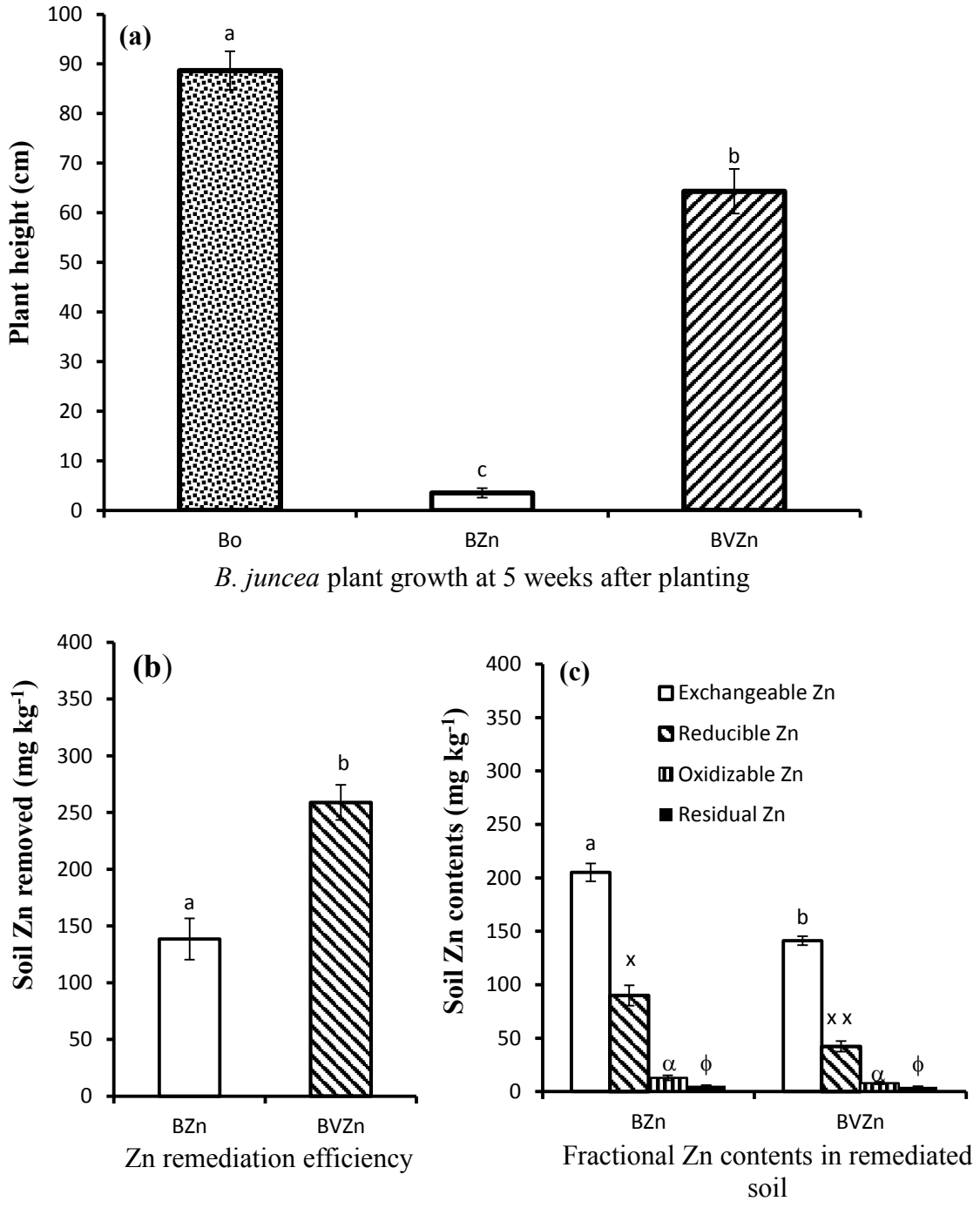



**Figure 6.6: (a) *B. juncea* plant height in uncontaminated soil (Bo), sole (BZn) and mixed (BVZn) planted under Zn contamination, (b) Zn removed per mass of soil and (c) amount of Zn in different fractions of remediated soil under BZn and BVZn plants. Bars are mean values at 5 weeks after planting and error bars show standard errors (n=3). Different letters/symbols indicate significant ($p<0.05$) differences between treatments. Figure shows *V. sativa* improves plant growth and Zn phytoremediation by *B. juncea***

The Zn concentration removed from the contaminated soil was significantly higher in mixed planted *B. juncea* (BVZn) than in sole planted *B. juncea* (BZn) treatment (Figure 6.6b). BVZn plants also remediated significantly more exchangeable and reducible soil Zn than BZn plants (Figure 6.6c). It is noteworthy that the higher Zn remediation observed under the mixed planting system (mean = 259 mg $kg^{-1}$) exceeds the sum of the individual remediating effects of the two plants (mean = 199 mg $kg^{-1}$, Figure 6.2). The observed plant growth promotion and enhanced Zn remediation in the mixed planting system is therefore attributed to the biochemical processes induced by the presence of the leguminous plant.

Although there is no sufficient data with which to evaluate the mechanistic basis for the observed growth promotion, the plant growth promoting activities of diverse bacteria species associated with nodules or roots of leguminous plants have been recognized as key processes responsible for better yield in crops co-planted with legumes (Dakora, 2003, Akhtar et al., 2013a). Plant growth promoting rhizobacterium and other microbes have been shown to enter roots of non-leguminous host plants through cracks or points of lateral root emergence and to establish themselves as endophytes in the xylem and intercellular spaces of host plants (Dakora, 2003). Although not tested in this experiment, it is likely that the better growth and remediation observed in BVZn plants is induced by the diverse bacteria population associated with the leguminous *V. sativa* plants. This interpretation is possible because in order to replicate natural conditions, a sterile condition was not maintained in the glasshouse and it is expected that natural rhizobia will colonise the roots of the leguminous plant.

It has been demonstrated that co-planting with a leguminous plant to promote the growth of *B. juncea* under Zn contamination (BVZn, mean plant height after 5 weeks = 64 cm) completely out performs the plant growth promoting effects of bacteria (BRZn and BPZn, mean plant height after 5 weeks = 29 and 17 cm, respectively) in Zn contaminated soil. Nevertheless, plant height in the BVZn treatment is still significantly lower than *B. juncea* plants grown in uncontaminated soil (Bo, mean plant height after 5 weeks = 89 cm). Hence, in the final component of this study, a strategy that combines the use of PGPB with legume co-planting on *B. juncea* plant growth and phytoremediation efficiency in Zn contaminated soil was explored.

### 6.3.4. Combining PGPBs with mixed planting

A weekly analysis of plant growth under the experimental treatments shows that the growth of plants inoculated with *R. leguminosarum* and co-planted with *V. sativa* (BRVZn) was not affected by Zn contamination (as compared to the Bo treatments) from the first week till the end of the experiment (Figure 6.7). Moreover, the combined use of a PGPB and co-planting with the leguminous *V. sativa* (BPVZn and BRVZn treatments) promoted the growth of *B. juncea* plants in Zn contaminated soils significantly more than any other treatments 5 weeks after planting.

Remarkably, there was no significant difference between *B. juncea* plant heights in the BPVZn and BRVZn treatments and plants grown in uncontaminated soil (Bo) at 5 weeks after planting. As a result, BPVZn and BRVZn plants exhibited significantly higher Zn remediation efficiencies (Figure 6.8a) and remediated more exchangeable soil Zn than the PGPB and mixed planting only treatments (Figure 6.8b).

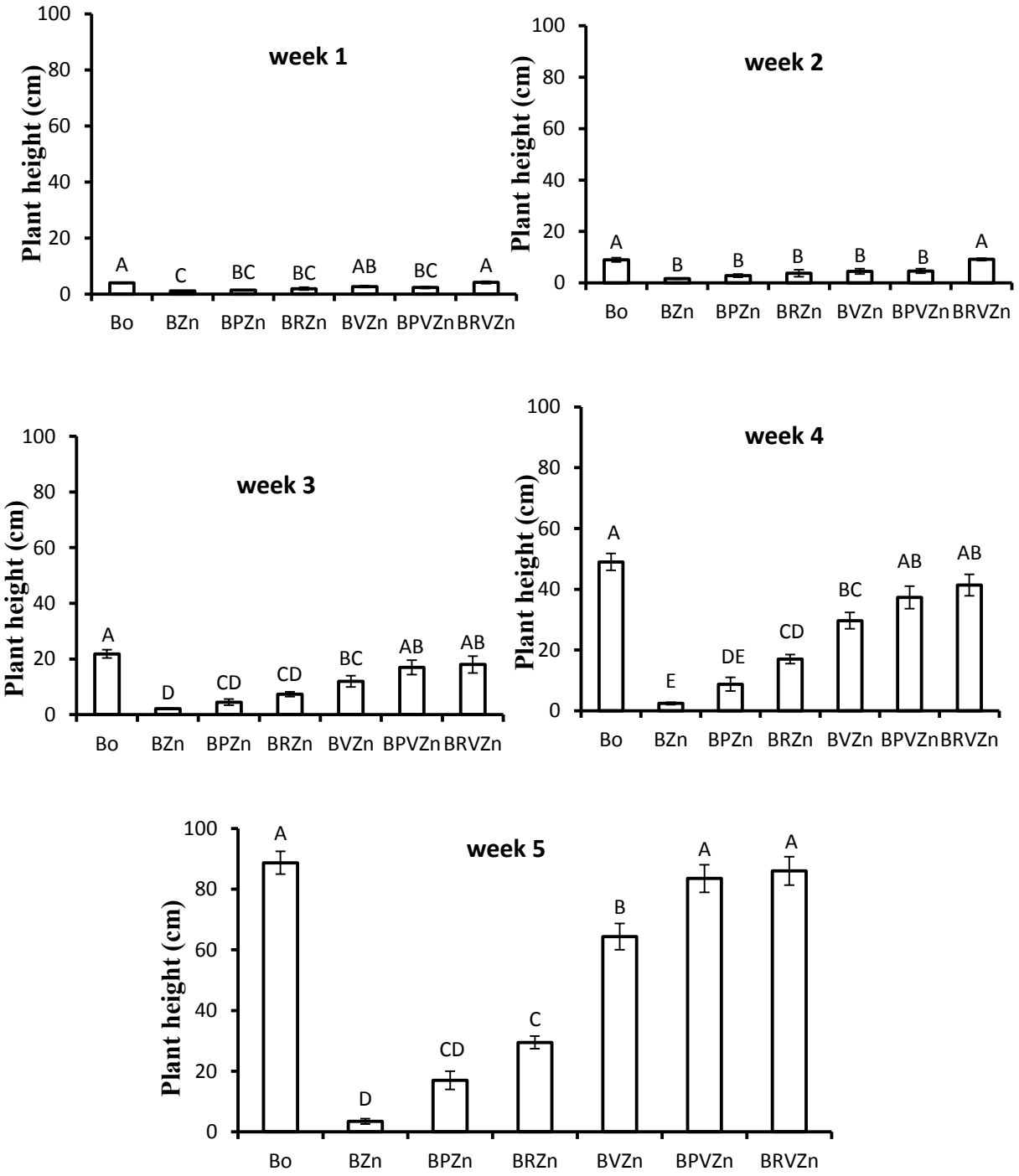



**Figure 6.7: Weekly comparison of *B. juncea* plant height in uncontaminated soil (Bo) with height under Zn contamination in sole planted (BZn), bacteria inoculated (BPZn & BRZn), mixed planted (BVZn), inoculated & mixed planted (BPVZn & BRVZn) *B. juncea*. Bars are mean plant heights from the 3 experimental pots and error bars show standard errors. Different symbols indicate significant ($p<0.05$) differences in weekly plant height between treatments (n=3). Figure shows that the growth of plants inoculated with *R. leguminosarum* and co-planted with *V. sativa* (BRVZn) was not significantly affected by Zn contamination from the first week till the end of the experiment**

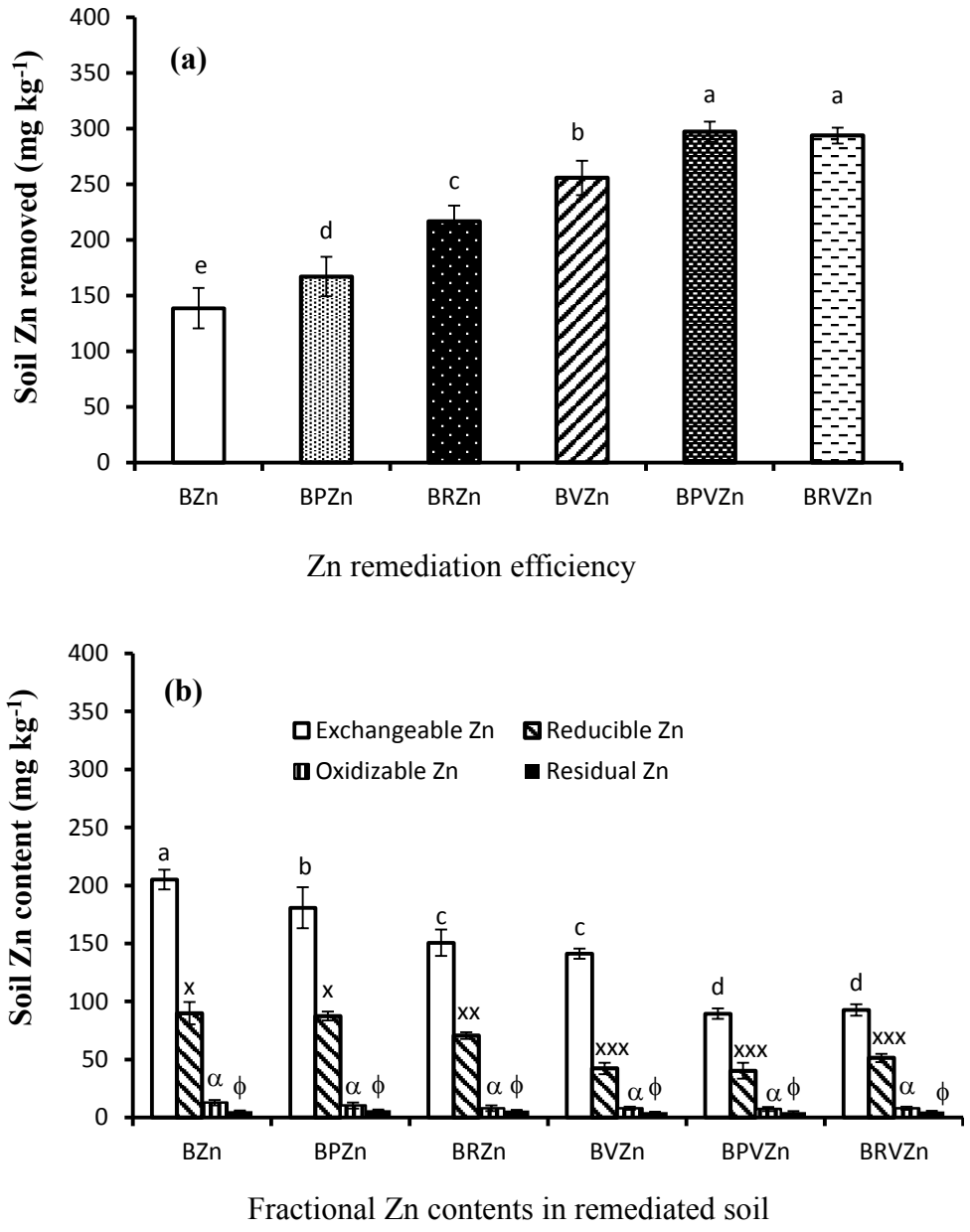



**Figure 6.8: (a) Zn removed per mass of soil, and (b) amount of Zn in different fractions of remediated soil under BZn, BPZn, BRZn, BVZn, BPVZn and BRVZn plants. Bars are mean values at 5 weeks after planting and error bars show standard errors (n=3). Different letters/symbols indicate significant ($p<0.05$) differences between treatments. Figure shows BPVZn and BRVZn plants exhibited significantly higher Zn remediation efficiencies and remediated more exchangeable soil Zn than the PGPB and mixed planting only treatments.**

It is clear that the BPVZn and BRVZn treatments combine the benefits of direct seed inoculation with PGPB with the plant growth promoting effects of the leguminous *V. sativa* plants. The legume assisted-microbial phytoremediation method that is

reported in this Chapter is the first to demonstrate complete growth recovery in plants exposed to 400 – 450 mg $kg^{-1}$ soil Zn contamination for 5 weeks.

### 6.3.5. Mechanisms behind observed Zn tolerance and remediation

The biochemical mechanism behind the observed poor Zn tolerance but better remediation in *B. juncea*, in contrast to the Zn hypertolerance but poor remediation in *V. sativa*, was investigated through a synchrotron based XAS analysis of root biomass from BZn and VZn plants. μXRF imaging of Zn in the plant root showed that *B. juncea* accumulated significantly more Zn than *V. sativa* (Figure 6.9), consistent with the relative differences in soil remediation efficiencies of the two plants.

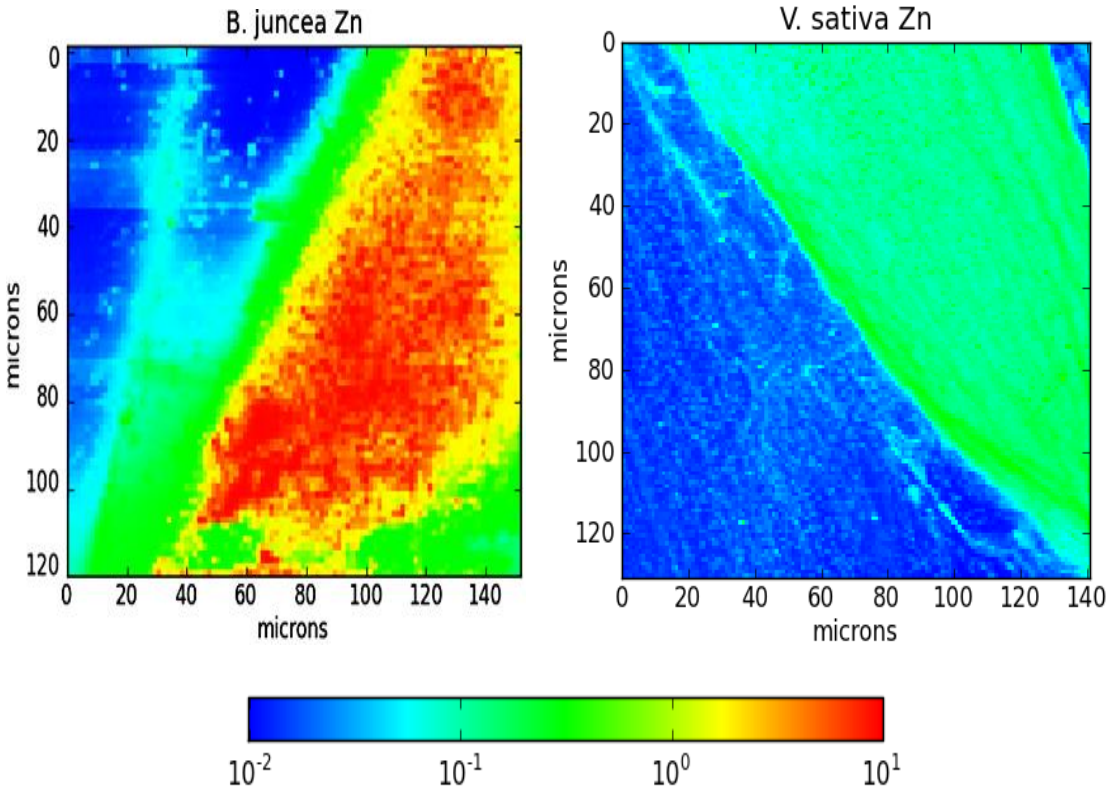



**Figure 6.9: Synchrotron based μXRF images of Zn distribution in the root of *B. juncea* and *V. sativa* plants from the BZn and VZn treatments. Zn counts are normalized to incoming beam intensity and the beam detector was at the same distance from the sample for the acquisition of the maps. Colour bars ($log_{10}$ scale) indicate Zn counts in plant roots from lowest (blue) to highest (red). Figure shows higher Zn bioaccumulation in *B. juncea* than in *V. sativa***

In addition to differences in the amount of Zn accumulation, the spatial distributions of Zn in the root of the two plant species are also conspicuously different. In *B. juncea* accumulated Zn appears to have been transported away from the cell walls and deposited in high amounts inside the root endodermis, probably in the root vascular tissue. Zn in *V. sativa* root on the other hand is more uniformly distributed throughout the root system. As a first line of defence against metal toxicity, uptake by the plant root is often reduced or prevented by a number of mechanisms, including binding metals to the root cell walls, restricting the metals to the apoplast (extracellular spaces between the cell walls), or cellular exudation of accumulated metals to the rhizosphere (Horst et al., 2010, Manara, 2012, Dalvi and Bhalerao, 2013).

The stunted growth observed in BZn plants is likely due to the accumulation of high amounts of Zn in the inner root tissue where metal sensitive organelles are located (Dalvi and Bhalerao, 2013). The better growth but low metal accumulation observed in *V. sativa* plants under Zn contamination may be due to biochemical restriction of the accumulation of high metal concentration at the plant epidermis or through cellular exudation.

Moreover, μXANES analysis of Zn speciation shows conspicuous differences in normalized Zn K edge, an indication of differences in Zn speciation between the *B. juncea* and *V. sativa* root samples (Figure 6.10a). Zn speciation was determined through LCF of Zn μXANES spectra in the root samples.

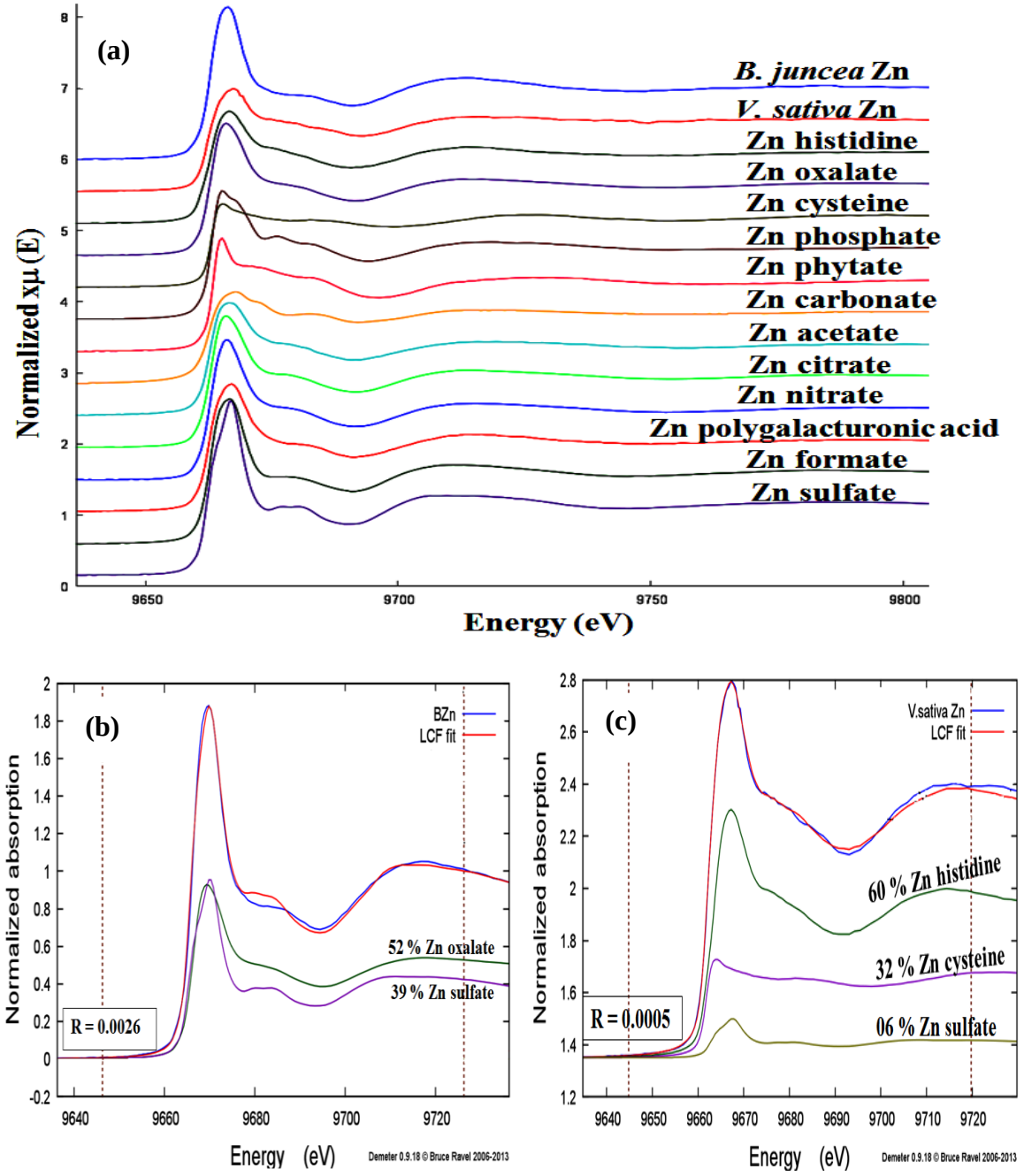



**Figure 6.10 (a) Normalized XANES of Zn in *B. juncea* and *V. sativa* plant roots and selected model Zn compounds, Zn K-edge XANES fitting, R factor and % Zn compound composition for (b) *B. juncea* (BZn) and (c) *V. sativa* (VZn) plant roots. R = Σi (experimental-fit)$^2$ / Σi (experimental)$^2$. The lower the R value the better the fit. Figure shows distinct differences in the species of Zn in the root of *B. juncea* and *V. sativa***

BZn has a higher *R* value and the fit with all combinations of the chosen standards was not as good as VZn (Figure 6.10b-c), making it difficult to determine root Zn speciation with confidence in the BZn plants. The fit suggests that in BZn plants Zn is predominantly present as Zn oxalate (~52%) and Zn sulfate (~39%). However, there may be another Zn species present in BZn plant roots for which we do not have a standard spectrum, such as Zn malate which has been observed to accumulate in other phytoremediating plants (Sarret et al., 2002). This tentative interpretation of Zn speciation in the root of BZn plants is supported by other studies that showed Zn oxalate accumulation in the root of Zn-resistant ecotype *Silene cucubalus* and *Rumex acetosa* planted on Zn spiked nutrient medium (Mathys, 1977).

Secretion of organic acids and formation of organic acid-metal complexes have been observed to be important response mechanisms to metal toxicity in many plant species (Broadley et al., 2007). Oxalate is known to enhance the sequestration of Zn by forming stable Zn oxalate in root vacuoles, but high concentrations have been reported to increase Zn toxicity to plants (Mathys, 1977). Furthermore, Zn was added to the experimental pots in the form of inorganic Zn sulphate which appears as one of the dominant Zn species in *B. juncea* root after the 5 week growing period. The significant Zn phytoremediation observed under *B. juncea* is therefore attributed to plant accumulation of Zn mainly in the forms of oxalate and sulphate. Accumulation of these toxic Zn forms may subsequently be responsible for the stunted plant growth observed in our experiment, by providing a pool that is dynamic equilibrium with Zn in the extracellular fluid.

In contrast, in *V. sativa* root Zn accumulated mostly in the chelated forms of Zn histidine (~60%) and Zn cysteine (~32%) with small traces of Zn sulphate (~6%). Metallothioneins and phytochelatines that contain histidine and cysteine respectively are metal chelating proteins that have been reported to induce tolerance to metal toxicity in plants (Kinnersley, 1993, Blindauer, 2008). They protect plants from the deleterious effects of heavy metals through a number of mechanisms including: metal chelation and subsequent isolation of toxic metal from metal sensitive enzymes (Cobbett, 2000), subcellular metal compartmentalisation and sequestration in vacuoles (Yadav, 2010), mediation of toxicity induced by oxidative stress, and plasma membrane repair (Cobbett and Goldsbrough, 2002, Hall, 2002).

The Zn hyper-tolerance observed in *V. sativa* is therefore attributed to plant root accumulation of Zn as mostly Zn histidine and cysteine. Zn prefers O and N as ligands and binds more strongly to hard O- and N-containing ligands but only weakly to soft S-containing ligands (Kopittke et al., 2011). Sulphur rich compounds such as phytochelatins have been reported not to be involved in Zn phyto-accumulation (Kopittke et al., 2011). This factor, coupled with the spindle-like plant size (Figure 6.11) and very low biomass of the *V. sativa* plants may be responsible for the low Zn remediation efficiency observed in this experiment.

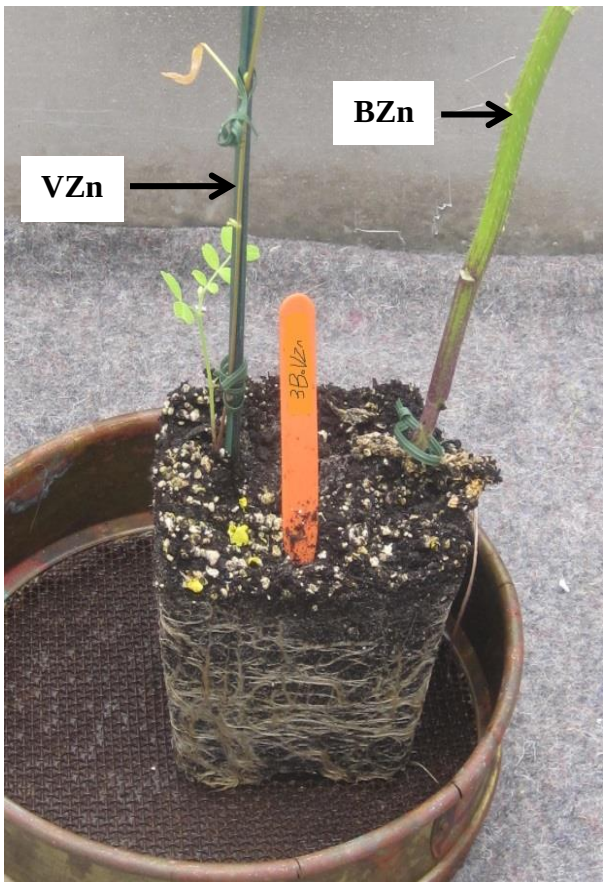



**Figure 6.11: *V. sativa* – *B. juncea* mixed planting system under Zn contamination at 5 weeks after seeds planting. Figure shows the spindle-like plant size of *V. sativa* plant and entwined roots of mixed planted *V. sativa* and *B. juncea* under Zn contamination**

Unfortunately, roots of *B. juncea* and *V. sativa* were completely mixed and entangled in the mixed-planting pots (Figure 6.11) such that it proved difficult to isolate *B. juncea* roots for XAS analysis and mapping, so that we could not confirm biochemically the extent of metal tolerance conferred by *V. sativa* through this mechanism. This was compounded by the limited availability of beam-time to analyze all the different treatments. Nevertheless, it is likely that the better plant growth and enhanced Zn phytoremediation ability of *B. juncea* co-planted with the

leguminous *V. sativa* is due to exogenous secretion of different types of beneficial metal chelates within the rhizosphere of the plant.

Both natural and synthetic chelates have been reported to significantly enhance the tolerance and remediation efficiency of phytoremediating plants (Tandy et al., 2006, Liu et al., 2008), for example, addition of EDTA to Zn contaminated soil in a pot experiment significantly increased Zn accumulation by *B. juncea* (Ebbs and Kochian, 1998). There are, however, serious environmental concerns about the application of synthetic chelating agents to remediate contaminated soils due to the potential drastic increase in metal solubility and bioavailability, and possible leaching losses to groundwater (Jiang et al., 2003).

### 6.3.6. Conclusion and wider environmental implications of result

As hypothesised, the Zn tolerant leguminous plant *V. sativa* conferred its inherent Zn tolerant ability to *B. juncea*; a plant with Zn phytoremediating potential but of poor tolerance to high soil Zn contamination, when both plants were established together in a legume-phytoremediator mixed planting system. Moreover, the legume-phytoremediator mixed planting system outperforms the use of PGPB in promoting the growth of *B. juncea* under Zn contamination and in enhancing Zn phytoremediation in Zn contaminated soil.

Furthermore, the form in which metal is stored in plant root is a key parameter in determining the metal tolerance and metal accumulating abilities in plants. Although Zn stored in the chelated forms of Zn histidine and cysteine helps the survival ability

of *V. sativa* under Zn contamination it reduces its ability to accumulate metal. On the other hand, Zn predominately in the form of Zn oxalate and sulphate in *B. juncea* appears to favours high metal accumulation but are likely to be responsible for the stunted growth of the plant under Zn contamination.

The use of synthetic chelates in enhancing toxic metal phytoremediation is gaining attention, but care must be taken to prevent potential secondary environmental contamination associated with the remediation method (Nowack et al., 2006). Conversely, this Chapter reports a novel method that uses a leguminous plant which secretes natural chelate to enhance Zn phytoremediation. Although the use of a PGPB with the leguminous plant conferred the best Zn phytoremediation effect, the legume-phytoremediator mixed planting system is recommend as a cheaper and simpler remediation alternative. The scope of the research however needs expanding to screen more leguminous plants for chelate secretion and to evaluate various combinations of tolerant leguminous species co-planted with phytoremediating plant species under many toxic metal contaminants.

Apart from changes in Zn speciation in roots, conspicuous differences in the nature of Zn sequestration in the roots of un-inoculated *B. juncea* and *V. sativa*, and in plants inoculated with the PGPB has been observed but it is not clear if this also defines tolerance and Zn accumulation in these treatments. It also not clear if the nature of bacterial colonisation influence the nature of Zn sequestration in the root of plant inoculated with PGPB and exposed to Zn contamination. Chapter 7 will therefore examines sub-cellular Zn accumulation and speciation in the epidermis and endodermis of *B. juncea* root, with and without inoculation with PGPB.

Chapter 7

# 7. Bacteria - Zinc co-localisation and Zinc speciation in *Brassica juncea* roots: a combined fluorescence microscopy and XAS analysis

## 7.1. Introduction

Zinc is a constituent of many enzymes and proteins that sustain life, and is thus an essential trace metal needed for optimum growth and development in plants (Broadley et al., 2007, Hafeez et al., 2013). It is, however very toxic to plants at elevated concentrations, with plants exhibiting toxicity symptoms like stunted roots, curling and rolling of young leaves and older leaves, chlorosis and necrosis (Chaney, 1993, Rout and Das, 2003). Plant leaf accumulation of < 100 mg Zn $kg^{-1}$ dry weight (DW) generally hinders normal plant growth, although the majority of susceptible plants normally exhibit visible toxicity symptoms of leaf chlorosis and necrosis at $[Zn]_{leaf}$ > 300 mg Zn $kg^{-1}$ leaf DW (Chaney, 1993, Broadley et al., 2007). In the plant cell, condensation of chromatin materials, disruption of cortical cell organelles and dilation of nuclear membranes has been reported under cytoplasmic concentration of 7.5 μM Zn (Rout and Das, 2003). Zn toxicity in plants has been described as the most extensive microelement phytotoxicity in nature (Chaney, 1993).

Different levels of Zn tolerance have been demonstrated to exist among different plant species (Shen et al., 1997, Ebbs and Kochian, 1997) and different mechanisms through which plants develop tolerance to elevated concentrations of Zn have been suggested (Baker, 1987, Clemens, 2001). For example, the organelles of plant cells have been observed to respond differently to elevated concentrations of Zn (Clemens, 2001, Hall, 2002). The binding of Zn to more tolerant organelles of the root cells like

the cell wall and cell membrane, in order to reduce the concentration of Zn that other Zn sensitive organelles in the root endodermis are exposed to, has been suggested as a possible strategy of Zn tolerance in plant roots (Lang and Wernitznig, 2011, Krzesłowska, 2011).

Moreover, the vacuole has been shown to be the main plant organelle responsible for storing toxic compounds in plant cells (Clemens, 2006, Wu et al., 2010). Rapid efflux of Zn from the cytosol into the vacuoles, and subsequent vacuolar sequestration of excess Zn in a process literally described as 'arrest and imprison' has also been suggested as a possible mechanism of Zn tolerance in plants (Clemens, 2001, Kobae et al., 2004, Wu et al., 2010). Apart from sub-cellular compartmentalisation of Zn, reduction of Zn bioavailability in plant cells through Zn complexation by phytochelatins and metallothioneins has also been suggested as a key mechanism behind plant tolerance to elevated Zn concentrations (Cobbett and Goldsbrough, 2002, Hossain et al., 2012).

Although previous results from metal tolerance studies have been useful in providing insight into the mechanism of metal tolerance in plants, most of the studies used intrusive chemical methods to extract cellular contents with little or no information on the specific region of the plant cell (whether from the vacuole or the cell wall) where the extracts were coming from (Brune et al., 1994, Hartley-Whitaker et al., 2001). Studies using imaging have only provided metal distribution in plant roots with little or no information about sub-cellular metal complexation (Küpper et al., 2000, Tian et al., 2014), whereas studies of metal speciation in whole plant organs have not been useful in providing adequate information about metal detoxification mechanisms at the sub cellular level (Kopittke et al., 2011, Song et al., 2013).

Moreover, the use of plant growth promoting bacteria (PGPB) to confer tolerance to plants exposed to toxic concentrations of Zn is gaining attention (Burd et al., 1998, Wani et al., 2007). Some of these PGPB have been reported to increase Zn bioaccumulation while enhancing plant growth, a process that may be further explored in using plants of less agronomical importance for detoxifying Zn contaminated environment (Li et al., 2007, Long et al., 2013). Most of these PGPB often colonise plant roots and are closely associated with root epidermis, the external plant regions including the root rhizosphere, rhizosplane and or the root cortex (Compant et al., 2010, Dimkpa et al., 2014). It has however not been demonstrated if PGPB enhance plant tolerance to Zn through enhanced metal compartmentalization at the cell epidermis, or the vacuole, or whether they attenuate toxicity through bacteria induced Zn chelation.

This study therefore uses a combination of fluorophore based confocal laser scanning microscopic imaging with synchrotron based microscopic X-ray fluorescence imaging and X-ray Absorption Spectroscopy, to study the nature of bacterial localisation, Zn accumulation, distribution and speciation in the roots of *B. juncea* established with and without *Pseudomonas brassicacearum*, *Rhizobium leguminosarum* and a combination of the two bacterial strains, in Zn contaminated media established under sterile experimental conditions.

For the microscopic analysis described in this research, the plant root was broadly studied as two main cellular regions: (i) the epidermis, the area of plant root extending from the cell wall from about 5.0 μm into the cortex and (ii) the endodermis, the inner cell area in the membranes of the endodermis including the vacuole and the vascular bundles (Di Laurenzio et al., 1996).

It was hypothesised that:

(i) There will be changes in Zn speciation between the epidermis and endodermis of the plant root due to the differences in the nature and properties of the root cell wall and the vacuole.

(ii) The PGPB will co-localise with Zn at the root epidermis and enhance epidermal Zn sequestration, since higher root exudation and bacterial population are mostly at the root epidermis compared to the root endodermis (el Zahar Haichar et al., 2008, Compant et al., 2010).

(iii) There will be differences in the nature of bacteria-metal co-localisation between *P. brassicacearum* and *R. leguminosarum* since the former is a native endophytic bacterial strain of Brassica roots and the latter is a rhizospheric bacterial strain isolated from the root of a clover plant.

(iv) Zn speciation at the epidermis and endodermis of inoculated root will be different due to possible more bacterial-metal co-localisation at the root epidermis than the endodermis

## 7.2. Experimental

### 7.2.1. Materials

*Brassica juncea*, a well-known accumulator of Zn was used in this experiment. *Pseudomonas brassicacearum* isolated from the root of a Brassica plant, and *Rhizobium leguminosarum* isolated from the nodules of a Clover plant were used as plant growth promoting bacteria. These strains were selected for their ability to colonise plant roots, promote plant growth and enhance Zn accumulation in *B. juncea* plants as previously reported in Chapter 3 and 4.

The growth medium used for the seed germination and seedling growth was Sterile Tork advanced wiper 420 centerfeed roll M2 System placed in sterile petri dishes. This seed germination and seedling growth method is similar to the roll towel test (Ma et al., 2011b) and the plant growth promotion assay on filter paper developed by (Glick et al., 1995) and modified by (Belimov et al., 2001). The method has been used to assess plant root growth promotion activities of bacteria in inoculated *B. juncea* plants exposed to cadmium toxicity (Belimov et al., 2005) and in *Orychophragmus violaceus* plants exposed to Zn (He et al., 2010). The media was able to sustain *B. juncea* plant growth for 14 days after seed planting without the need of nutrient supplements. Zn sulphate was used as the source of Zn contamination. The experiment was conducted under a sterile laminar flow cabinet at 25°C with artificial lightning used to simulate day/night photoperiods.

### 7.2.2. Experimental treatments

A completely randomised design of 8 treatments (Table 7.1), each comprising 8 replicates, was randomly distributed in the sterile cabinet. Plant growth parameters were assessed from 6 replicates while the remaining 2 replicates were used for XAS analysis. For each of the experimental treatments, 5g of sterile wiper paper (folded as 2.5 g into the base and cover of the petri dishes respectively) were placed into sterile petri dishes (see more details in Chapter 3). The paper filled petri dishes were then exposed to UV light for 30 min to ensure the death of any bacteria that might have contaminated the system during the folding process. For treatments that required Zn contamination, Zn sulphate solution was prepared with sterile deionised water, filtered through a 0.22 mm filtration unit to ensure the solution was free of contamination from bacteria and applied to the paper at the rate of 400 mg $kg^{-1}$.

**Table 7.1: Description of experimental treatments**

| Treatment | Description |
|---|---|
| B*o* | *B. juncea* plants, un-inoculated, in media not contaminated by Zn |
| B*Po* | *B. juncea* plants inoculated with *P. brassicacearum* |
| B*Ro* | *B. juncea* plants inoculated with *R. leguminosarum* |
| B*RPo* | *B. juncea* plants inoculated with *P. brassicacearum* and *R. leguminosarum* |
| BZn | Un-inoculated *B. juncea* plants under Zn contamination |
| B*P*Zn | *B. juncea* plants inoculated with *P. brassicacearum*, under Zn contamination |
| B*R*Zn | *B. juncea* plants inoculated with *R. leguminosarum* , under Zn contamination |
| B*RP*Zn | *B. juncea* plants inoculated with *P. brassicacearum* and *R. leguminosarum*, under Zn contamination |

The pH of Zn contaminated paper determined in deionised water was 6.4-7.2 which means the possibility of plant toxicity due to acidity was low in the experiment and the pH of the Zn solution was therefore not adjusted. The volume of sterile deionised water that corresponded to the volume of Zn solution added to Zn contaminated treatments was added to controls without Zn to ensure all treatments started with the same moisture contents.

For treatments that required bacteria inoculation, seeds of *B. juncea* were surface sterilised with 0.05 M sodium hypochlorite, washed 3 times in sterile deionised water and soaked under aseptic conditions in a bacteria-sterile water suspension of $7.5 \times 10^8$ CFU $ml^{-1}$ at 30°C for 3 hours. Seeds for treatments without bacteria inoculations were soaked in sterile deionised water for the same duration under the same experimental conditions. For each treatment, 12 seeds were placed in a 4 x 3

grid pattern in the paper filled petri dishes. The treatments were kept moist with sterile deionised water throughout the experiment.

### 7.2.3. Seed germination and seedling growth assessment

In this study, seeds with visible radicle (about 1 mm) emerging from the seed coat were regarded as germinated seeds (Munzuroglu and Geckil, 2002, Kopyra and Gwóźdź, 2003). Numbers of seeds that germinated were counted at 14 days after planting and percentage seed germination was calculated as: (Gs/Ts)*100, where, Gs and Ts are number of seeds that germinated and total number of seed planted respectively. Fresh roots and shoot lengths were measured and total dry biomass of seedlings in each plate was estimated by drying biomass at 70°C to a constant weight and weighing with a sensitive balance. Zn tolerance indices were calculated separately for roots, shoots and the whole seedling as: (LZn/Lo)*100, where LZn and Lo are shoot/root length and total dry biomass in media contaminated with Zn and uncontaminated media respectively.

### 7.2.4. Preparation of plant roots for microscopic imaging

In order to ensure the acquisition of images of bacteria and Zn in the internal cell structure of plant roots at high resolution, thin sections of 35 μm thickness of root biomass were acquired through the use of a microtome under cryogenic conditions. Root strands were gently harvested and immediately cryo-fixed in Optimum Cutting Temperature compound (OCT) at -80°C before cryo-sectioning. OCT is a viscous water soluble gel composed of non-reactive ingredients, polyvinyl alcohol and polyethylene glycol (Turbett and Sellner, 1997, Weston and Hummon, 2013). Cryo-fixing the samples in OCT helped to preserve the structural integrity of the sample

and made the sample easy to handle during the cutting process (Knapp et al., 2012). Cryo-embedded samples were smoothly sectioned into uniform sections with a Leica CM1900 Cryostat at a cryostat temperature of -35 to -25ºC. Sectioned samples for microscopic imaging were collected on standard microbiology glass slides while samples to be analysed by XAS were collected on sterile Kapton tapes placed on glass slides and clipped into place with an adhesive tape. The adopted biomass sectioning method ensured minimum disturbance of bacteria/metal association and distribution. Sterility was maintained throughout the sample preparation process.

### 7.2.5. Confocal Laser Scanning Microscopy (CLSM)

Sectioned root samples on the standard glass slides were stained with a nucleic acid stain SYTO®9 and a metallic ion stain RhodZin™-3 for bacteria and Zn imaging respectively. The green fluorescing SYTO9 stain is capable of penetrating all types of bacterial cytoplasmic membrane and it is used to assess total bacteria population (Leuko et al., 2004, Berney et al., 2007). On the other hand, RhodZin-3 is an orange fluorescent indicator for $Zn^{2+}$ molecule (Sabnis, 2010, Wiederschain, 2011) capable of penetrating cell membranes and it is gaining recognition as a valuable fluorophore for investigating the physiological consequences of $Zn^{2+}$ in living cells (Kikuchi et al., 2004, Bonanni et al., 2006). Both stains do not contain any toxic metal and are water soluble at room temperature (Sabnis, 2010, Wiederschain, 2011, Leuko et al., 2004). SYTO 9 has a maximum Excitation/Emission spectra of 484/498 nm (Stocks, 2004) and RhodZin-3 has a maximum Excitation/Emission spectra of 549/576 nm (Sabnis, 2010) making it possible to combine the two for simultaneous staining and imaging of Zn and bacterial cells in plant biomass.

0.83 μg $mL^{-1}$ of SYTO9 and 5.0 μg $mL^{-1}$ of Rhodzine-3 were prepared with sterile deionised water under aseptic conditions, and plant samples were stained singly and in combinations with the two stains, and incubated in the dark for 30 minutes before microscopic imaging. Stained slides were covered with 20 mm x 50 mm no. 1.5 cover slides and sealed with nail polish. A Zeiss Axio imager was used to screen and select the best samples, and the selected samples were imaged with a Leica SP5 Confocal Laser Scanning Microscope (CLSM). For all the samples imaged with the CLSM, at least 12 micrometer thick z-stacks images were acquired at a 0.5 μm z-interval. This image acquisition method affords a step-wise study of sample cross-sections along the sample's depth. Serially collected images were then reconstructed into 3 – dimensional images using Image J software.

### 7.2.6. Micro X-ray Fluorescence Spectroscopy (μ-XAS)

Sectioned plant samples collected on Kapton tapes were preserved in dry ice and transported to the Diamond Light Source UK for μ-XRF mapping on the microfocus beamline I18. All samples were analyzed within three days of harvesting from Zn contaminated media. The cryo-fixing method that was used to preserve the samples has been judged to be effective for preserving the nature of biological samples for up to 4 weeks (Knapp et al., 2012). Samples were mounted on the beam sample holder at an angle of 45° to the incident beam of 2.5 μm beam size. The beamline energy was calibrated using a Zn foil (9661 eV). Synchrotron micro X-ray fluorescence (μXRF) data of the samples were collected in fluorescence mode using a nine-element germanium solid state detector. The beam detector was at the same distance from the sample for the acquisition of the Zn maps for all treatments. The collected μXRF data were processed into images using PyMCA 4.4.1 (Solé et al., 2007).

Points (6 at the root epidermis and 3 at the root endodermis) displaying high Zn concentrations were selected from the μXRF images for microfocus X-ray Absorption Near Edge Structure (μXANES) analysis. Consecutive spectra from the same point were examined for possible beam damage and only the best spectra were used for μXANES analysis. Each of the treatments was analysed in duplicates.

In order to determine the chemical composition of the collected μXANES spectra, Zn K-edge μXANES spectra were also collected under similar beam conditions for some selected Zn standards – Zn oxalate, Zn phosphate, Zn histidine, Zn cysteine, Zn phytate, Zn polygalacturonate, Zn formate, Zn sulfate, Zn nitrate, Zn citrate, Zn acetate and Zn carbonate. These include Zn phytochelatines, metalotheionines and other Zn complexes that have been observed to be involved in Zn dynamics within the metal-bacteria-plant system studied (Terzano et al., 2008, Kopittke et al., 2011). Zn standards were used in Linear Combination Fitting (LCF) using a least-squares algorithm of the sample μXANES spectrum from 9645.3 to 9725.3 eV. The fractional contribution of each of the analysed standard compounds to the Zn spectrum was assumed to be directly proportional to the fraction of Zn present in that form in the plant root (Terzano et al., 2008). The goodness of the fit was estimated by calculating the residual *R* factor of the fit; $R = \Sigma_i (\text{experimental-fit})^2 / \Sigma_i (\text{experimental})^2$. The sums (Σ) are over 103 data points as flattened mu (E). A lower *R* factor represents a better match between the fitted standard spectra and the sample spectrum (Terzano et al., 2008).

### 7.2.7. Statistical Analysis

Growth parameters were estimated per petri dish and means of the parameters from treatments replicated 6 times were calculated (n=6). For the μ-XAS analysis, two plants were analysed per treatment (n=2), comprising one plant selected from the two replicated dishes. The 6 Zn spectra collected from the epidermis and 3 from the endodermis were merged per treatment and means of percentage Zn compound compositions at the epidermis and endodermis of the two treatments were calculated. All treatment means were tested for normal distribution using Anderson-Darling's normality test. All means are of equal variance and are normally distributed (Table 7.2). One way analysis of variance followed by Tukey's HSD test ($p<0.05$) was used to identify significant differences between treatment means. All statistical analyses were conducted using Minitab 16 software (Minitab$^{TM}$ Inc., USA).

Table 7.2. Results of Anderson Darling's (AD) normality test for means of seed germination percentage root and shoot length, seedling biomass and tolerance indexes. All treatment means are normally distributed (P-value > 0.05)

| Treatments | | AD-value | P-value |
|---|---|---|---|
| Germination percentage | B | 0.305 | 0.408 |
| | BP | 0.201 | 0.320 |
| | BR | 0.289 | 0.447 |
| | BRP | 0.206 | 0.339 |
| | BZn | 0.239 | 0.341 |
| | BPZn | 0.244 | 0.351 |
| | BRZn | 0.233 | 0.391 |
| | BRPZn | 0.389 | 0.520 |
| Root length | B | 0.221 | 0.512 |
| | B | 0.251 | 0.410 |
| | BP | 0.189 | 0.631 |
| | BR | 0.255 | 0.399 |
| | BRP | 0.189 | 0.631 |
| | BZn | 0.267 | 0.360 |
| | BPZn | 0.209 | 0.547 |
| | BRZn | 0.191 | 0.622 |
| Shoot length | BRPZn | 0.204 | 0.565 |
| | B | 0.221 | 0.512 |
| | BP | 0.238 | 0.320 |
| | BR | 0.255 | 0.399 |
| | BRP | 0.134 | 0.385 |
| | BZn | 0.293 | 0.292 |
| | BPZn | 0.258 | 0.388 |
| | BRZn | 0.259 | 0.384 |
| Total biomass | BRPZn | 0.272 | 0.302 |
| | B | 0.185 | 0.347 |
| | BP | 0.174 | 0.354 |
| | BR | 0.295 | 0.288 |
| | BRP | 0.233 | 0.32 |
| | BZn | 0.195 | 0.605 |
| | BPZn | 0.253 | 0.404 |
| | BRZn | 0.207 | 0.553 |
| Root tolerance index | BZn | 0.267 | 0.360 |
| | BPZn | 0.209 | 0.547 |
| | BRZn | 0.191 | 0.622 |
| | BRPZn | 0.204 | 0.565 |
| Shoot tolerance index | BZn | 0.293 | 0.295 |
| | BPZn | 0.258 | 0.388 |
| | BRZn | 0.259 | 0.384 |
| | BRPZn | 0.272 | 0.302 |
| Biomass Tolerance index | BZn | 0.195 | 0.605 |
| | BPZn | 0.253 | 0.404 |
| | BRZn | 0.207 | 0.553 |
| | BRPZn | 0.192 | 0.619 |

## 7.3. Results

### 7.3.1. Effect of PGPB on seed germination

There were no differences between the germination percentage of un-inoculated seeds and seeds inoculated with PGPBs at 14 days after planting in media without Zn contamination (Figure 7.1).

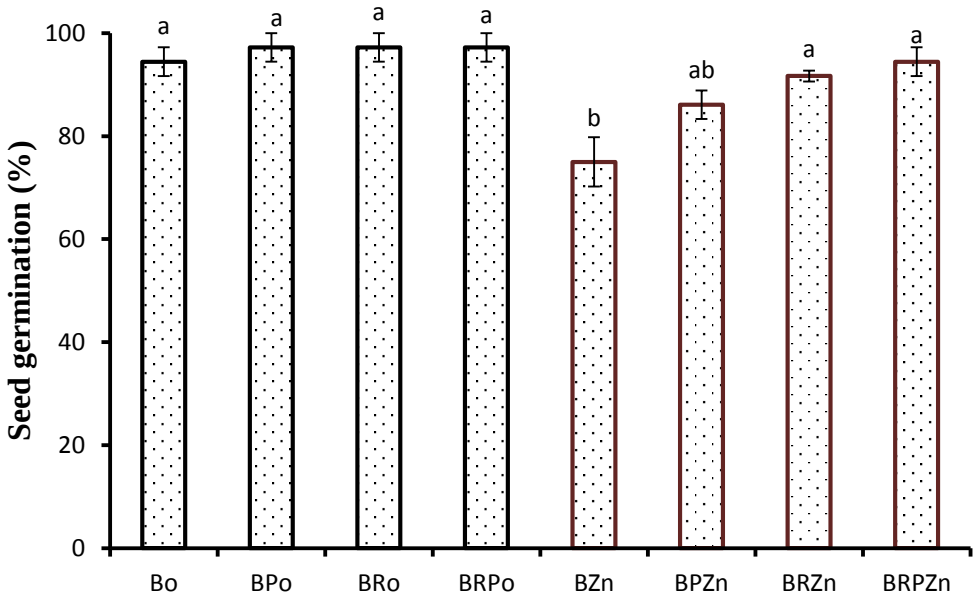



**Figure 7.1: Germination of *B. juncea* seeds un-inoculated (B), inoculated with *P. brassicacearum* (BP), *R. leguminosarum* (BR) and combinations (BRP) in uncontaminated media and under Zn contamination. Bars are mean (n=6) of seed germination percentage from 6 petri dishes each containing 12 seeds (total of 72 seeds). Error bars show standard errors. Different letters indicate significant ($p<0.05$) differences in seed germination percentage between treatments. Figure shows bacterial inoculated seeds germinated better under Zn contamination than non-inoculated seeds (BZn).**

In Zn contaminated media however, germination percentage in seeds that were not inoculated with PGPB (BZn) was significantly lower compared to the seeds in un-contaminated media. Although the seed coat of most of the seeds in the BZn treatments appeared to be broken, the development of the radicles was hampered and radicle lengths < 1 mm at 14 days after planting. Although inoculation of seeds with

*P. brassicacearum* (BPZn) improved seed germination percentage under Zn, the effect was not statistically significant. By contrast, *R. leguminosarum* and its combination with *P. brassicacearum* significantly enhanced seed germination and appeared to help the seeds recover full germination under Zn contamination when compared to the germination rates in treatments without Zn contamination.

### 7.3.2. Effect of PGPB on growth

Root, shoot and total seedling growth was assessed 14 days after planting in uncontaminated and Zn contaminated media, and percentage tolerance indexes (TI) were calculated. In media without Zn contamination, the roots of bacterial inoculated treatments (BPo, BRo, and BRPo) were only slightly longer than the treatment without bacterial inoculation (Bo) and the plant growth promoting effects of the PGPB were not statistically significant (Figure 7.2a). However, under Zn contamination, root growth in the BZn treatments were stunted while there was better plant root growth in treatments inoculated with PGPB, especially in BRZn and BRPZn treatments.

The root tolerance of the BZn plants to the 400 mg $kg^{-1}$ of Zn contamination was estimated to be 30.0% (Figure 7.2b). Although inoculation with *P. brassicacearum* improves root tolerance to about 45.0% the effect was not statistically significant. In the presence of *R. leguminosarum* and combination of the two bacterial strains, root tolerance to Zn contamination was significantly enhanced from 30.0% to 69.0% and 90.0% respectively.

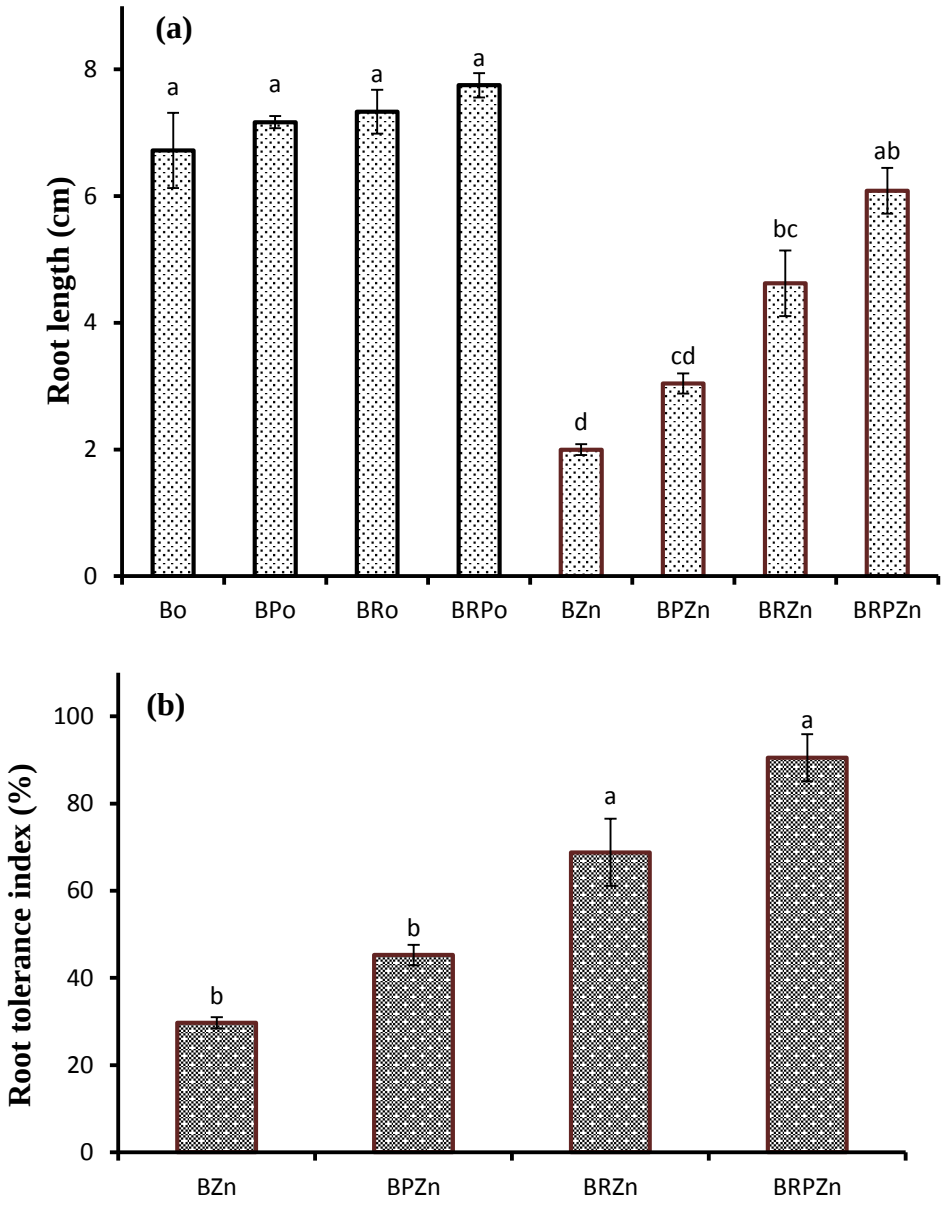



**Figure 7.2: (a) *B. juncea* root length un-inoculated (B), inoculated with *P. brassicacearum* (BP), *R. leguminosarum* (BR) and combinations (BRP) in uncontaminated media (o) and under Zn, and (b) Root tolerance index under Zn showing better root tolerance in treatment inoculated with *R. leguminosarum* and its combination with *P. brassicacearum*. Bars are means of (a) root length and (b) %TI from 6 petri dishes each containing 12 seeds. Error bars show standard errors. Different letters indicate significant ($p<0.05$) differences in root length and root TI between treatments.**

Furthermore, shoot growth in uncontaminated and Zn contaminated media was assessed. At 14 days after planting, the PGPB increased shoot length in uncontaminated media, but the effects were not statistically significant (Figure 7.3a).

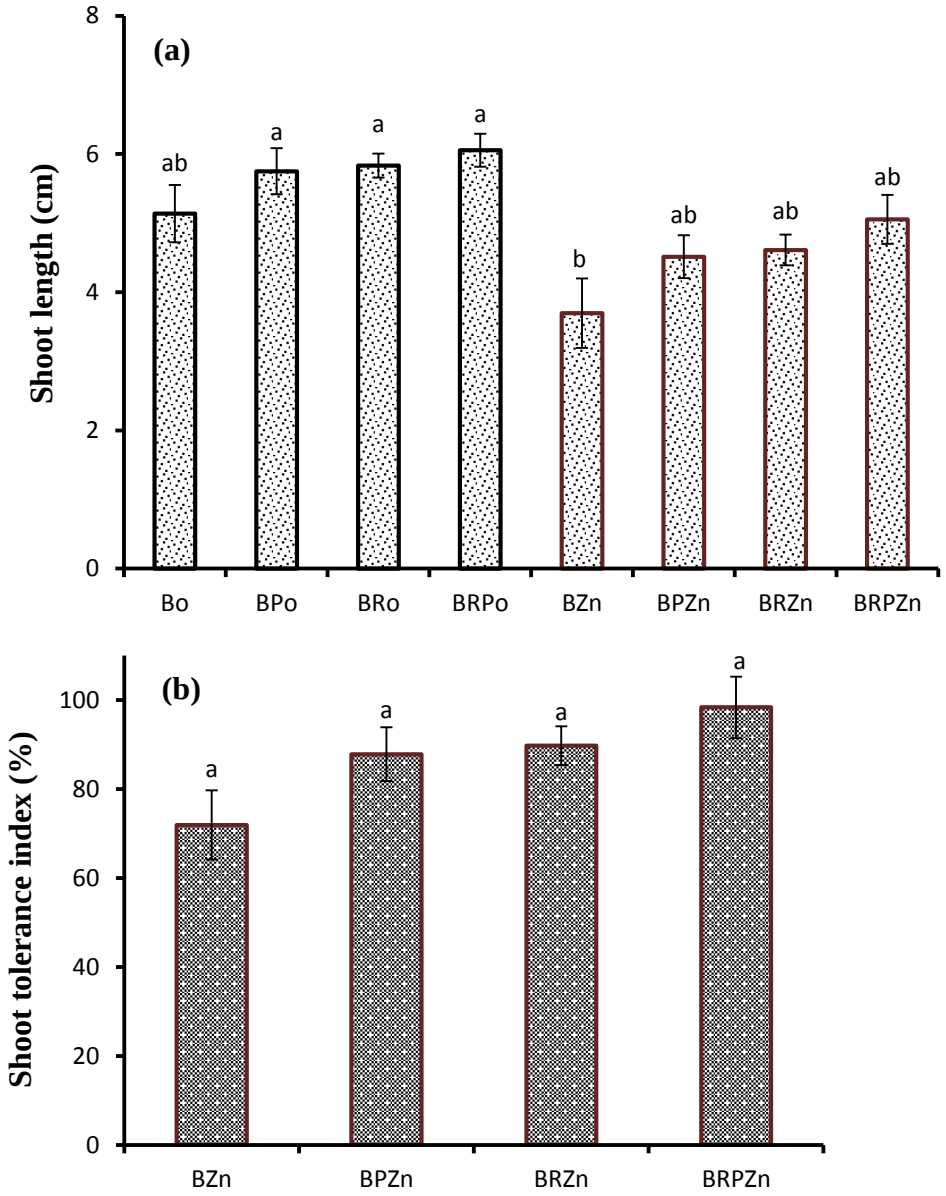



**Figure 7.3: (a) Shoot length in un-inoculated *B. juncea* (B), *B. juncea* inoculated with *P. brassicacearum* (BP), *R. leguminosarum* (BR) and combinations (BRP) growing in uncontaminated media (o) and under Zn and (b) Shoot tolerance index under Zn showing that shoot growth did not suffer from Zn contamination and the PGPBs did not have any significant effects on shoot growth. Bars are means of (a) shoot length and (b) %TI from 6 petri dishes each containing 12 seeds. Error bars show standard errors. Different letters indicate significant ($p<0.05$) differences in shoot length and root TI between treatments.**

Although shoot length was slightly reduced under Zn contamination, shoot length in BZn treatments was not significantly lower than the control (Bo) and was statistically the same with the BRZn, BPZn and BRPZn treatments. Moreover, there were no

significant differences in percentage shoot tolerance to Zn between treatments without bacterial inoculation (BZn) and the treatments with PGPB (BPZn, BRZn and BRPZn) (7.3b). Roots are the main organ for Zn extraction and it is likely that at this stage of seedling growth, most of the Zn is mainly in the root.

Furthermore, seedlings (roots and shoots) were harvested after the experiment and dry biomass was assessed. Although the highest amount of seedling biomass in un-contaminated media was in the BRPo treatments, seedling biomass was statistically the same in bacterial inoculated treatments and the control (Bo) (Figure 7.4a). Meanwhile, biomass in the BZn treatments was significantly reduced compared to biomass in uncontaminated media, and the treatments with PGPB exhibited better seedling growth under Zn contamination. It is worthy of note that seedling biomass in the BRZn and BRPZn treatment are the same as the biomass in the Bo treatment, an indication that seed inoculations with *R. leguminosarum* and its combination with *P. brassicacearum* helps the plant to recover full growth under Zn contamination.

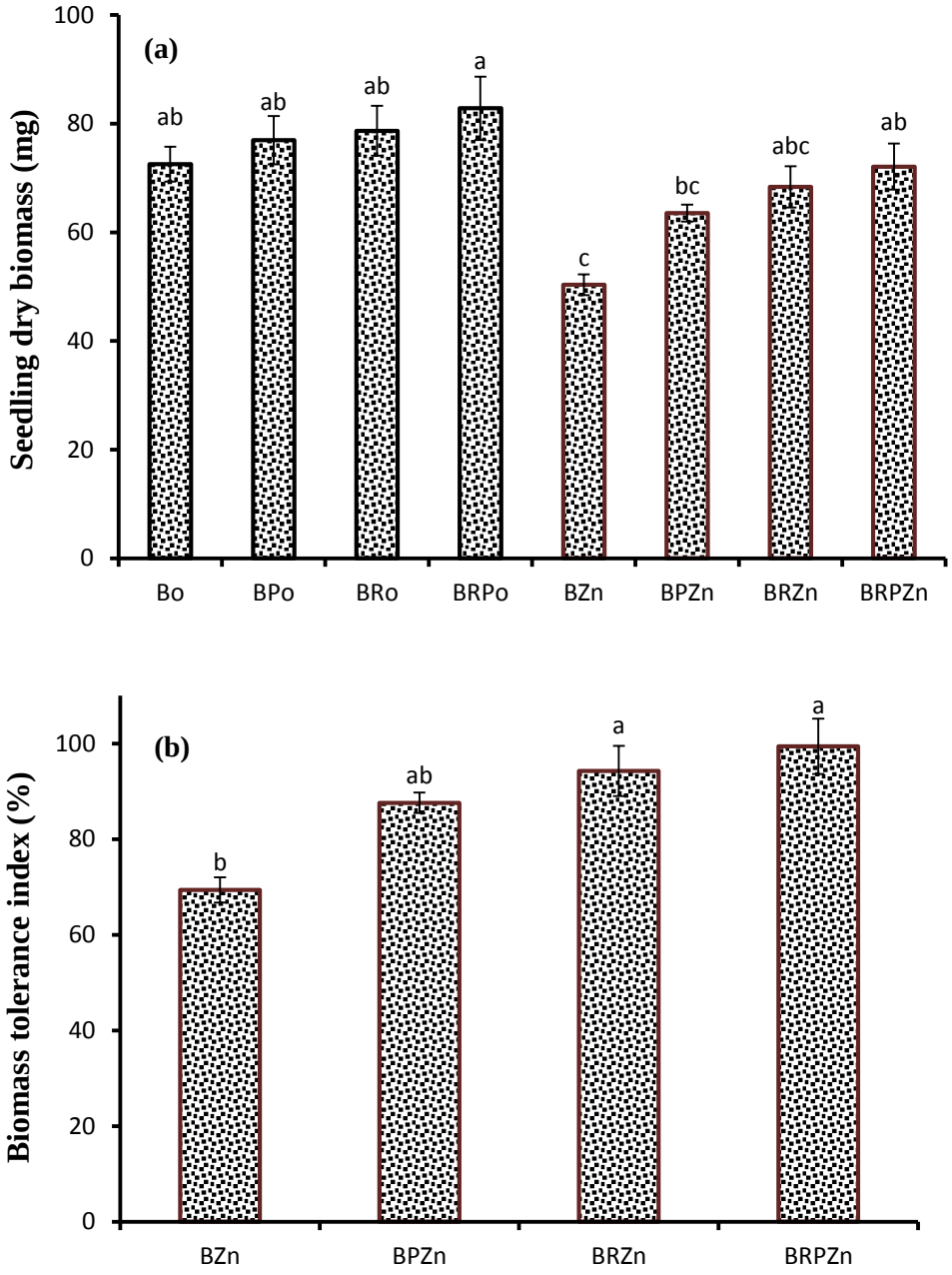



**Figure 7.4: (a) *B. juncea* dry seedling biomass un-inoculated (B), inoculated with *P. brassicacearum* (BP), *R. leguminosarum* (BR) and combinations (BRP) growing in uncontaminated media (o) and under Zn and (b) Biomass tolerance under Zn contamination showing significant biomass tolerance to Zn contamination in BRZn and BRPZn. Bars are means (n=6) of (a) dry seedling biomass and (b) %TI from 6 petri dishes each containing 12 seeds. Error bars show standard errors. Different letters indicate significant ($p<0.05$) differences in biomass weight and biomass TI between treatments.**

Moreover, although inoculation with *P. brassicacearum* improves the inherent Zn tolerance of *B. juncea* biomass from 69.4% to 87.6%, the most significant effects were observed under inoculation with *R. leguminosarum* (BRZn) and bacterial combinations (BRPZn) with TI of 94.3% and 99.4% respectively (Figure 7.4b). A plant that completely resists metal toxicity and exhibits its growth potential under metal contamination can be regarded to have a metal tolerance index of 100%. It can therefore be inferred, that inoculation of *B. juncea* seeds with a combination of *P. brassicacearum* and *R. leguminosarum* (with a TI of 99.4%) conferred complete tolerance to *B. juncea* seedlings established in Zn 400 mg $kg^{-1}$ for 14 days.

Furthermore, colonisation of plant root by bacteria strains and the ability of the bacteria to maintain a stable relationship with the root have been suggested as an important biological process for effective plant growth promotion by bacteria (Lugtenberg and Dekkers, 1999, Compant et al., 2005, Compant et al., 2010). Bacteria inoculation and colonisation of plant roots has also been observed to facilitate metal bioaccumulation. In order to understand the mechanism behind bacteria induced root tolerance to Zn contamination observed in this experiment, the localisation of the bacteria strains and Zn in the seedling root was examined 14 days after planting.

### 7.3.3. CLSM bacterial-Zn imaging in plant roots

A 3-D reconstruction of CLSM images of un-inoculated *B. juncea* root exposed to Zn contamination shows the absence of bacteria cells but evidence of Zn accumulation (Figure 7.5a). The roots of bacteria inoculated treatments (BPZn,

BRZn and BRPZn) on the other hand show evidence of bacterial colonisation of roots as well as Zn bioaccumulation (Figure 7.5b-d).

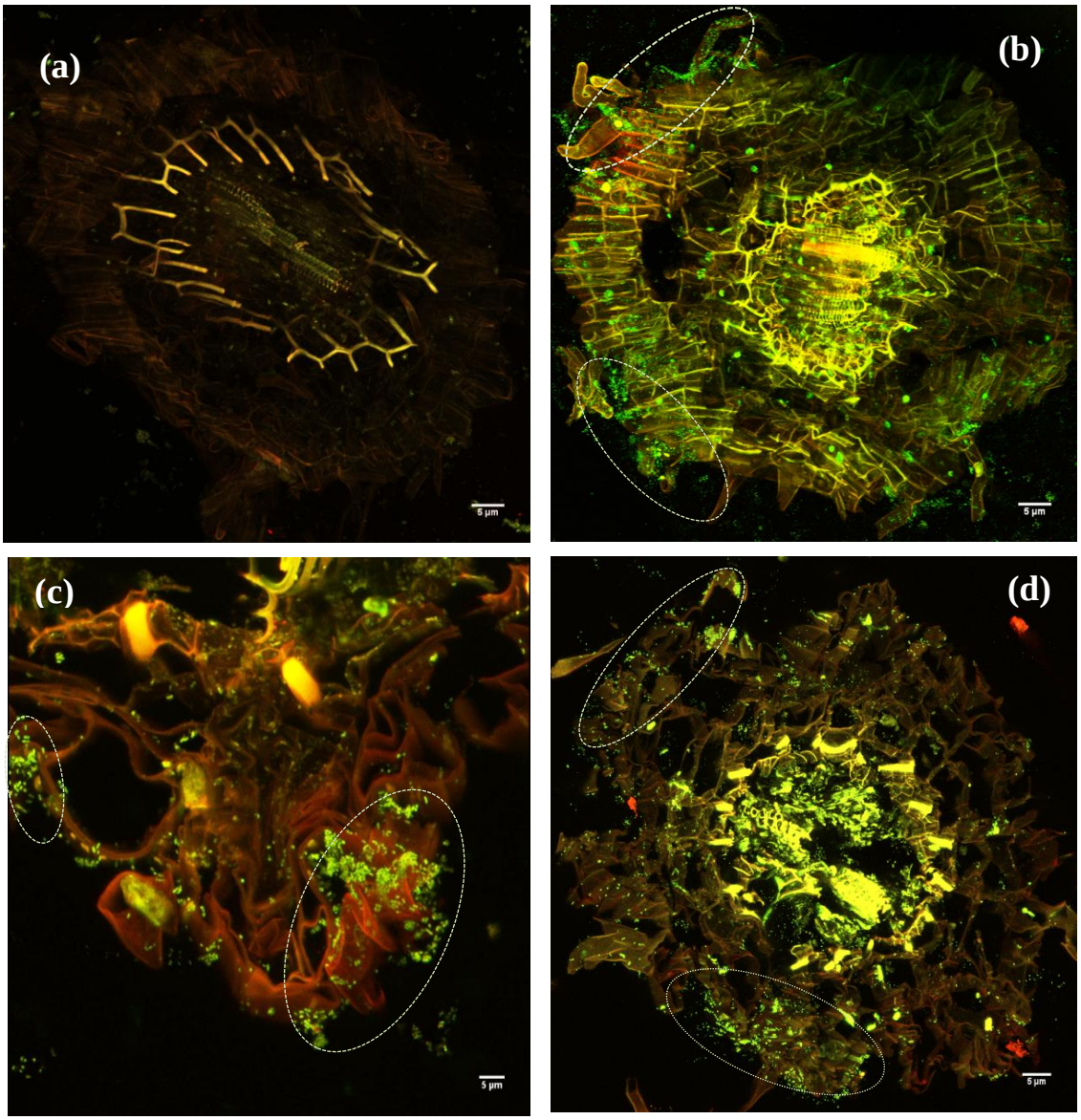



**Figure 7.5: 3-D reconstruction of CLSM images of *B. juncea* root un-inoculated (a), inoculated with *P. brassicacearum* (b), *R. leguminosarum* (c) and combinations of the two bacterial strains (d) 14 days after exposure to 400 mg $kg^{-1}$ Zn. Green fluorescent bodies are bacteria cells and the red spots are area of Zn localisation. Areas of high bacteria localisation along the root epidermis are circled. Figure shows absence of bacteria cells in the un-inoculated root**

To further understand the relationship between the bacterial strains and Zn accumulation within the root biomass, images of the densely localized areas were

acquired at a magnification of x100 (Figure 7.6 and 7.7). *P. brassicacearum* exhibited endophytic ability and appeared to colonise the interior of root strands despite the apparent root accumulation of Zn (see *S* in Figure 7.6).

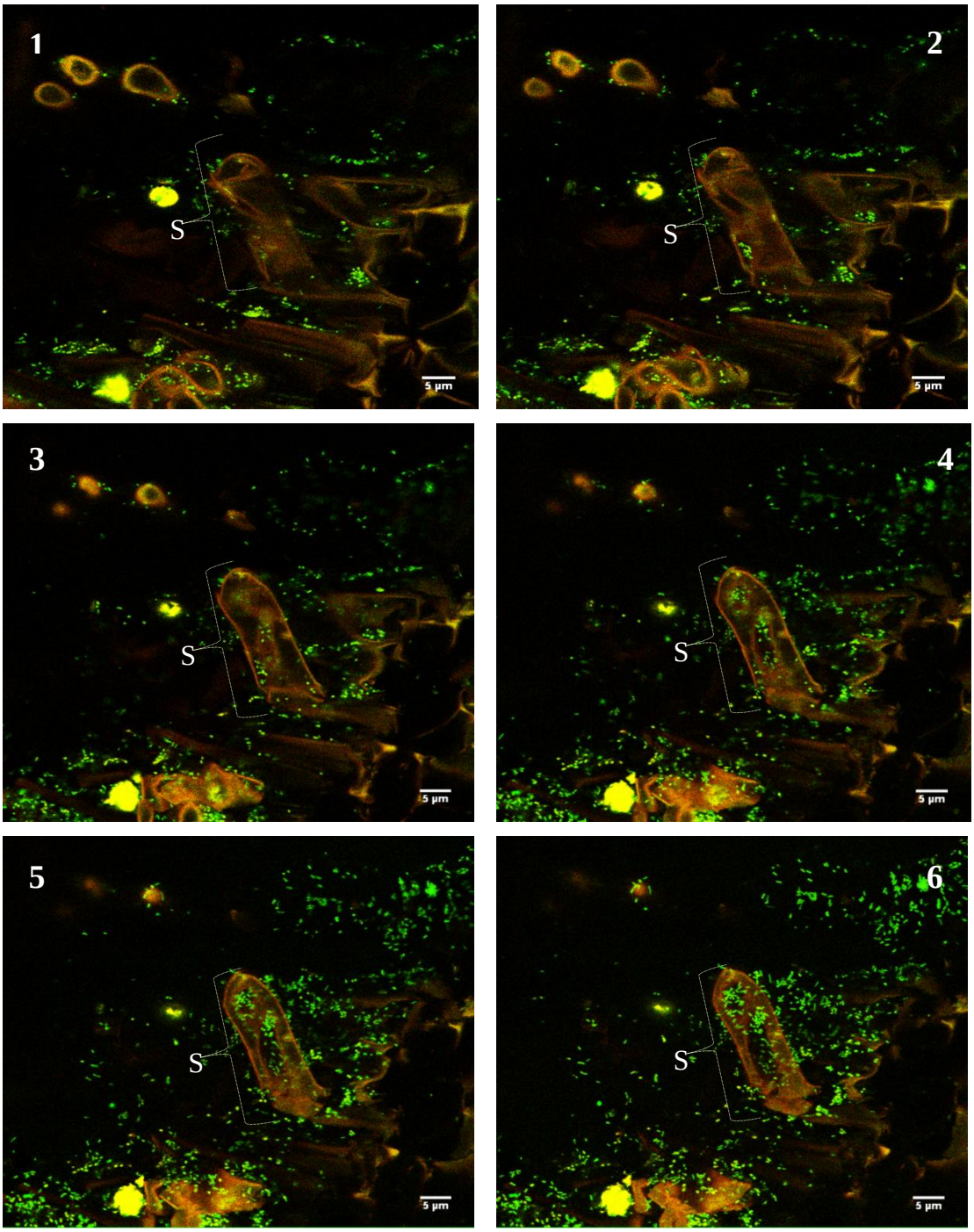



**Figure 7.6: CLSM imaging of *P. brassicacearum*-Zn co-localisation at the root cortex of *B. juncea*. *S* shows endophytic (interior) colonisation of a Zn embedded root strand. Numbers (1-6) shows sequence of image acquisition across the root depth. Green bodies are bacteria cells and Red spots indicate the presence of Zn**

Although *R. leguminosarum* appeared only to be residing in the outer spheres of the root, there are indications of bacteria colonisation at Zn concentrated areas (see *S* in Figure 7.7).

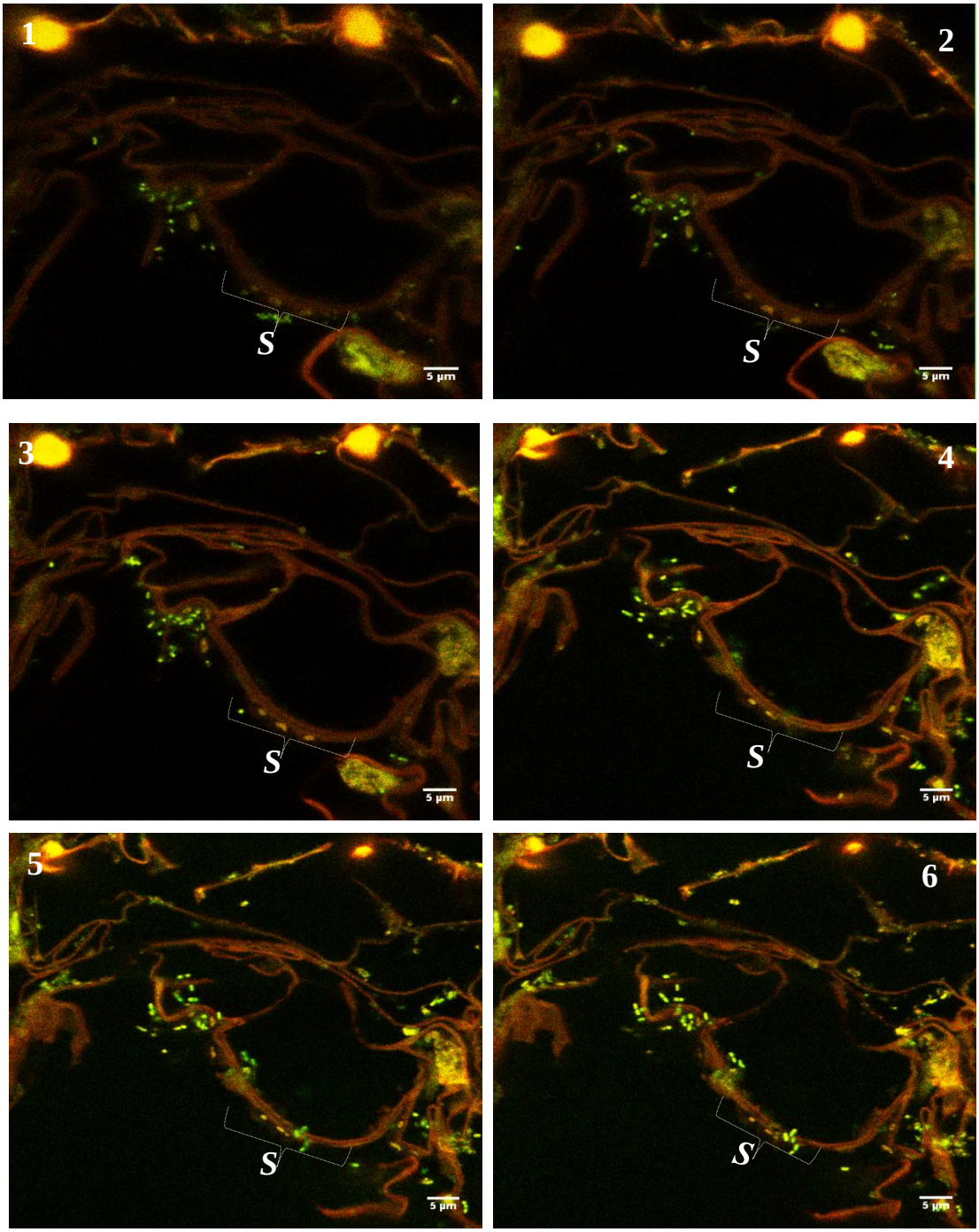



**Figure 7.7: CLSM imaging of *R. leguminosarum*-Zn co-localisation at the root epidermis of *B. juncea*. *S* shows bacterial colonisation at Zn localised areas. Numbers (1-6) shows the sequence of image acquisition across the root depth. Green bodies are bacteria cells and Red spots indicate the presence of Zn.**

In all the treatments, the bacterial strains appeared to only colonise the root epidermis with little presence of microbial biomass at the endodermis. To further investigate this, images of the root epidermis and root endodermis from the BRPZn treatments were acquired at x100 magnification and compared (Figure 7.8).

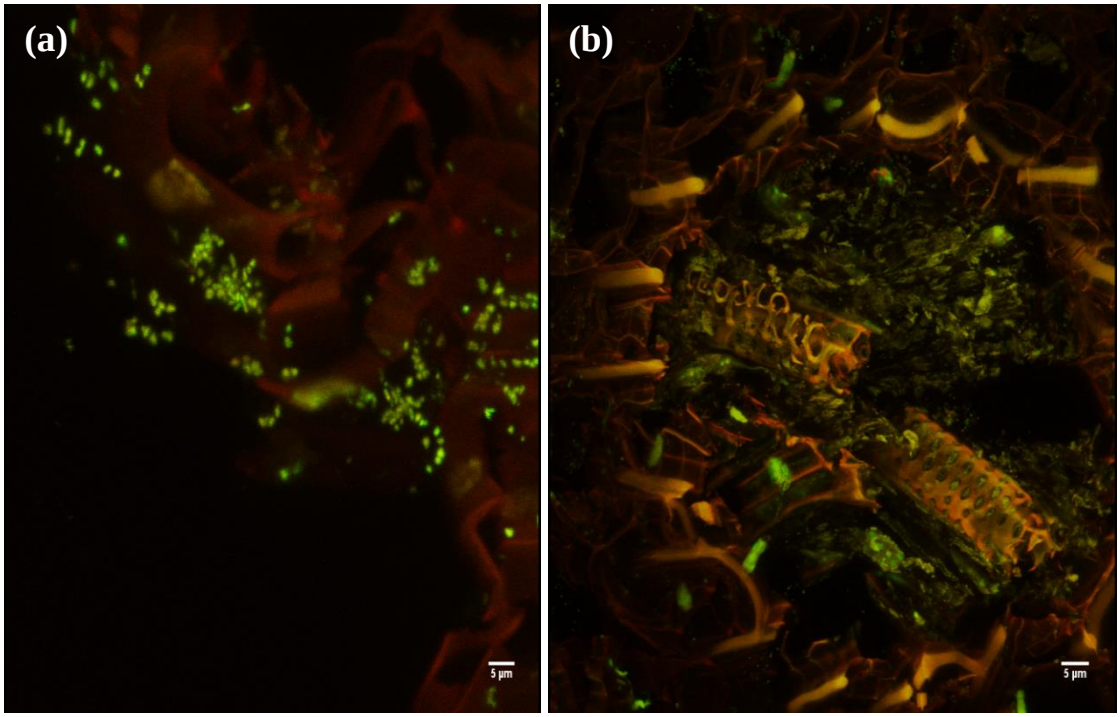



**Figure 7.8: 3-D reconstruction of root (a) epidermis and (b) endodermis in plant inoculated with *P. brassicacearum* and *R. leguminosarum*, and exposed to Zn for 14 days. Regular green bodies (< 5 µm) are bacteria cells and the red colour indicates Zn distribution. (a) Shows bacteria colonisation at the root epidermis while (b) shows the endodermis relatively free of bacterial cells (note: the green fluorescent bodies in (b) are from the DNA of plant cells not bacterial cells because they are larger (> 5 µm) than the size of bacteria.**

Analysis of the outer (Figure 7.8a) and inner (Figure 7.8b) root structures revealed bacteria colonisation at the root epidermis with the root internal structures relatively free of bacterial colonisation.

Although fluorophore based CLSM analysis was useful in understanding nature of bacterial colonisation under Zn contamination, a synchrotron based µ-XAS analysis

was needed to investigate possible changes in Zn accumulation and speciation due to the direct impacts of bacterial colonisation at the root epidermis and possible bacterial induced changes in the root endodermis, within inoculated treatments and in comparison to the treatments without bacterial inoculation.

### 7.3.4. μ-XAS analysis of Zn distribution and speciation

Results of synchrotron based μXRF imaging of Zn in plant roots revealed higher Zn bioaccumulation in bacteria inoculated plants than the un-inoculated plants, with the roots under dual bacteria inoculation exhibiting the highest Zn accumulation (Figure 7.9). There were also conspicuous differences in Zn accumulation pattern between the treatments. In the bacteria inoculated treatments (Figure 7.9b-d), more of the Zn was localised at the root epidermis in contrast to the un-inoculated treatment (Figure 7.9a) with a less defined Zn localisation pattern along the root epidermis. Moreover, there were conspicuous differences between Zn accumulation pattern under *R. leguminosarum* and *P. brassicacearum*. Whereas accumulated Zn in the BRZn (Figure 7.9c) exhibited a characteristic localisation pattern at the root epidermis with few high concentration points in the root endodermis, Zn accumulation along the epidermis in the BPZn treatments appeared to be more diffuse (Figure 7.9b) with more Zn in the endodermis than in the BRZn treatments.

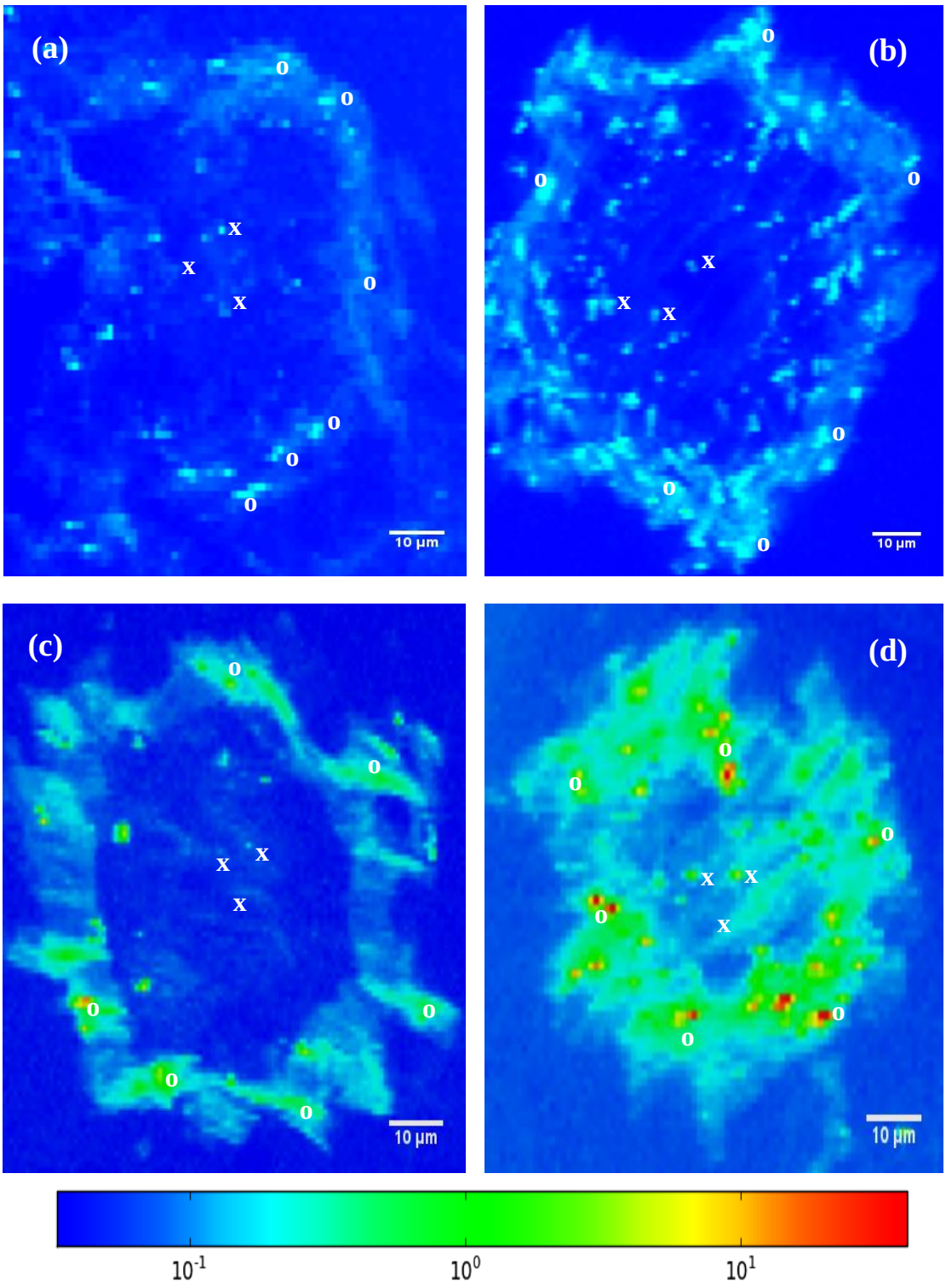



**Figure 7.9: Synchrotron µ–XRF imaging of Zn in the root of *Brassica juncea* un-inoculated (BZn) (a), inoculated with *P. brassicacearum* (b), *R. leguminosarum* (c) and combinations of the two bacterial strains (d) 14 days after seed planting in 400 mg $kg^{-1}$ Zn. Figure shows that the PGPB significantly enhance Zn sequestration at the epidermis. Symbol o and x represents spots in the root epidermis and endodermis respectively that were subjected to µ-XANES analysis.**

In all bacteria inoculated treatments where high Zn bioaccumulation in the seedling root was observed, better root growth was also recorded. This is counter intuitive, as higher Zn accumulation in root biomass should lead to reduced root growth. Possible differences in Zn speciation at the epidermis and endodermis of the treatments were therefore further investigated.

Principal component analysis of the treatment XANES spectra revealed Zn sulphate, Zn carbonate, Zn polygalacturonic acid, Zn oxalate, Zn cysteine and Zn phytate as the principal Zn species. The proportions of these principal Zn components in the XANES spectra was therefore estimated through LCF and R factors, a measure of the goodness of XANES fittings. Based on physical observation of the Zn k-edge XANES fits and R factor results that range from as low as 0.0004 to 0.0023, the results of LCF are good enough to sufficiently describe Zn speciation in the studied systems (Figure 7.10). Although there were no conspicuous differences in the structure of the treatment's XANES, there were statistically significant differences in the proportion of Zn-compound compositions among the treatments (Figure 7.11).

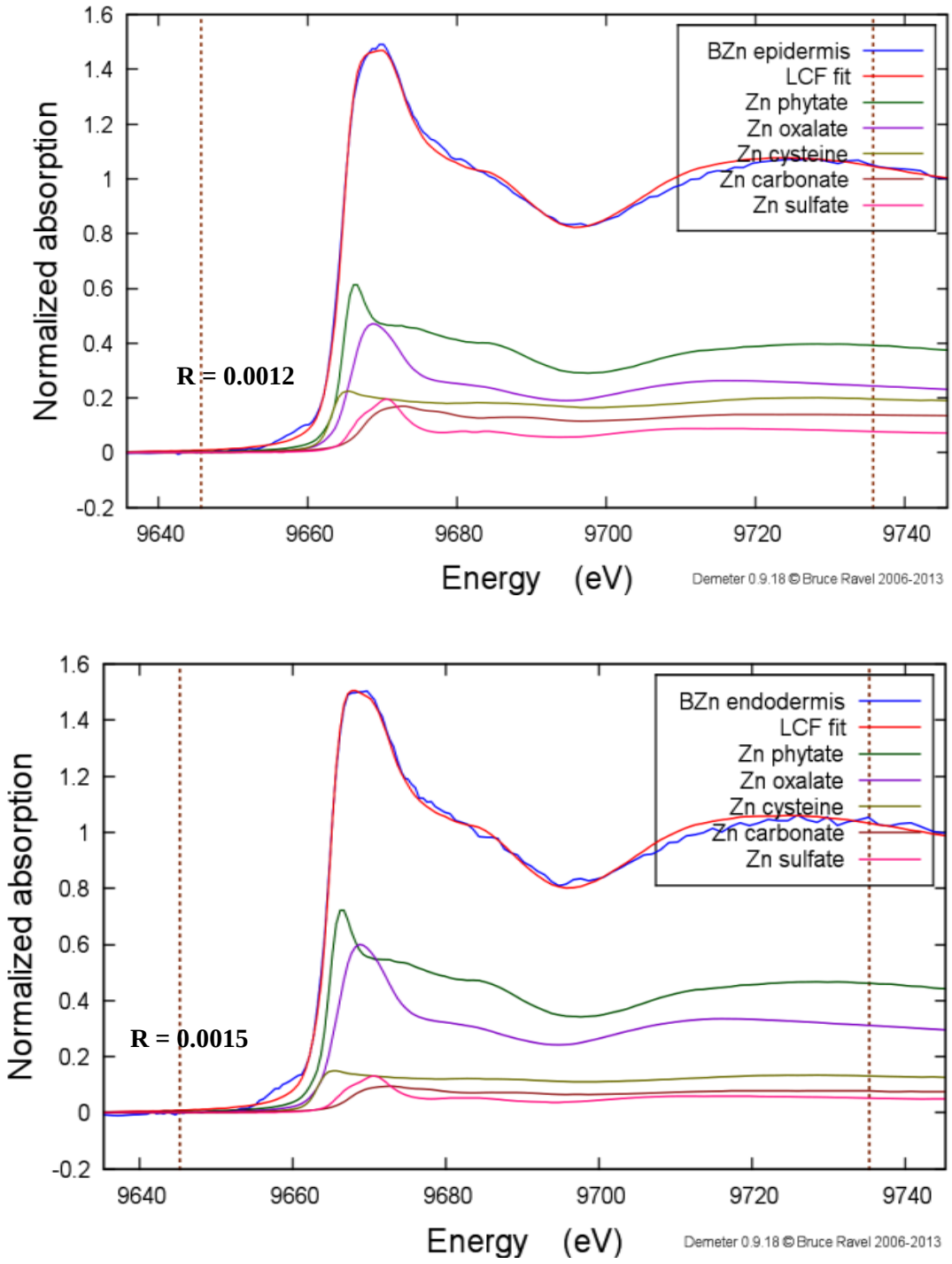
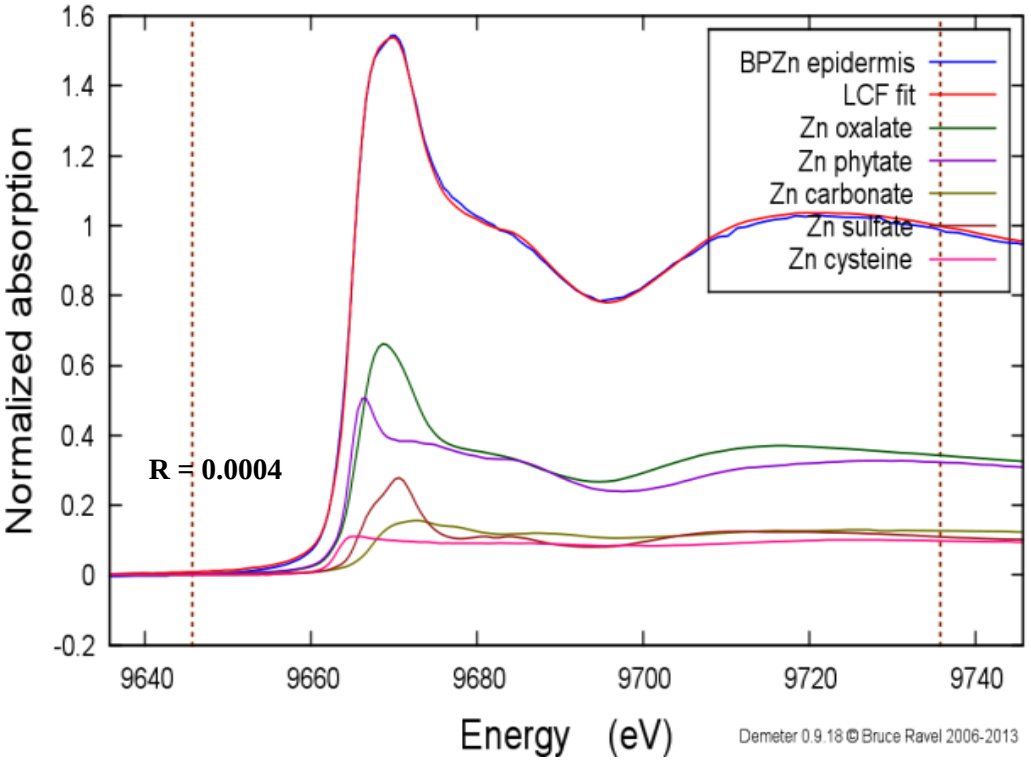



**Figure 7.10 (a): Zn K-edge XANES fitting, R-factors and Zn-compound compositions for epidermis and endodermis of un-inoculated root (BZn). *R* = Σi (experimental-fit)$^2$ / Σi (experimental)$^2$. The lower the *R* value the better the fit.**



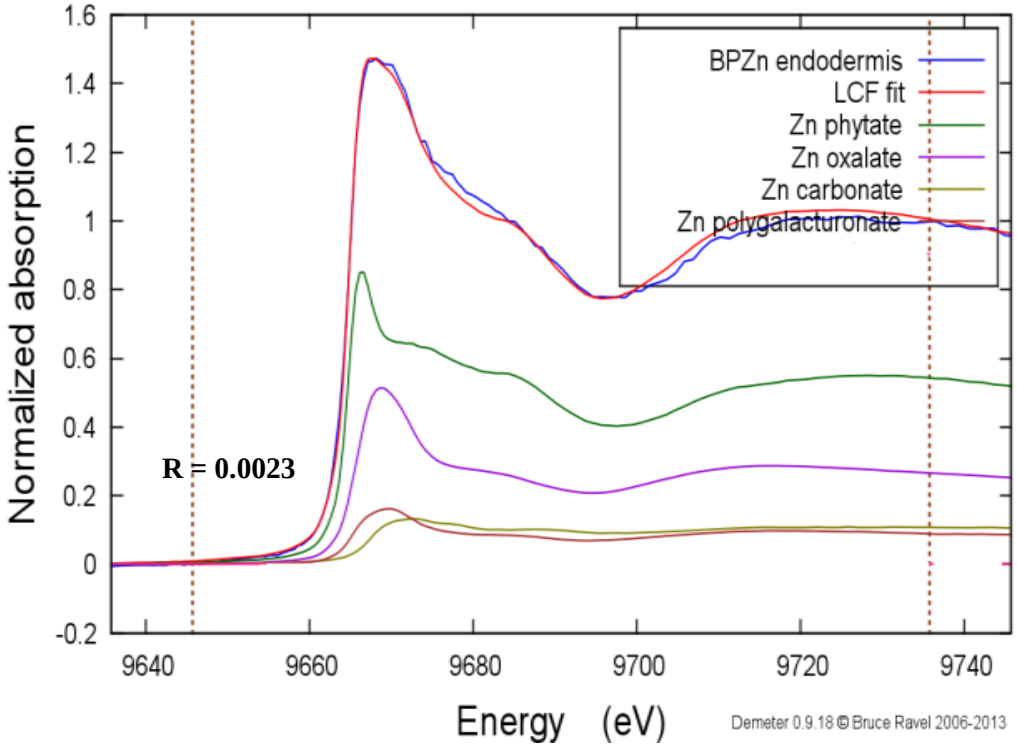




**Figure 7.10 (b): Zn K-edge XANES fitting, R-factors and Zn-compound compositions for epidermis and endodermis of root inoculated with *P. brassicacearum* (BPZn). *R* = Σi (experimental-fit)$^2$ / Σi (experimental)$^2$. The lower the *R* value the better the fit.**

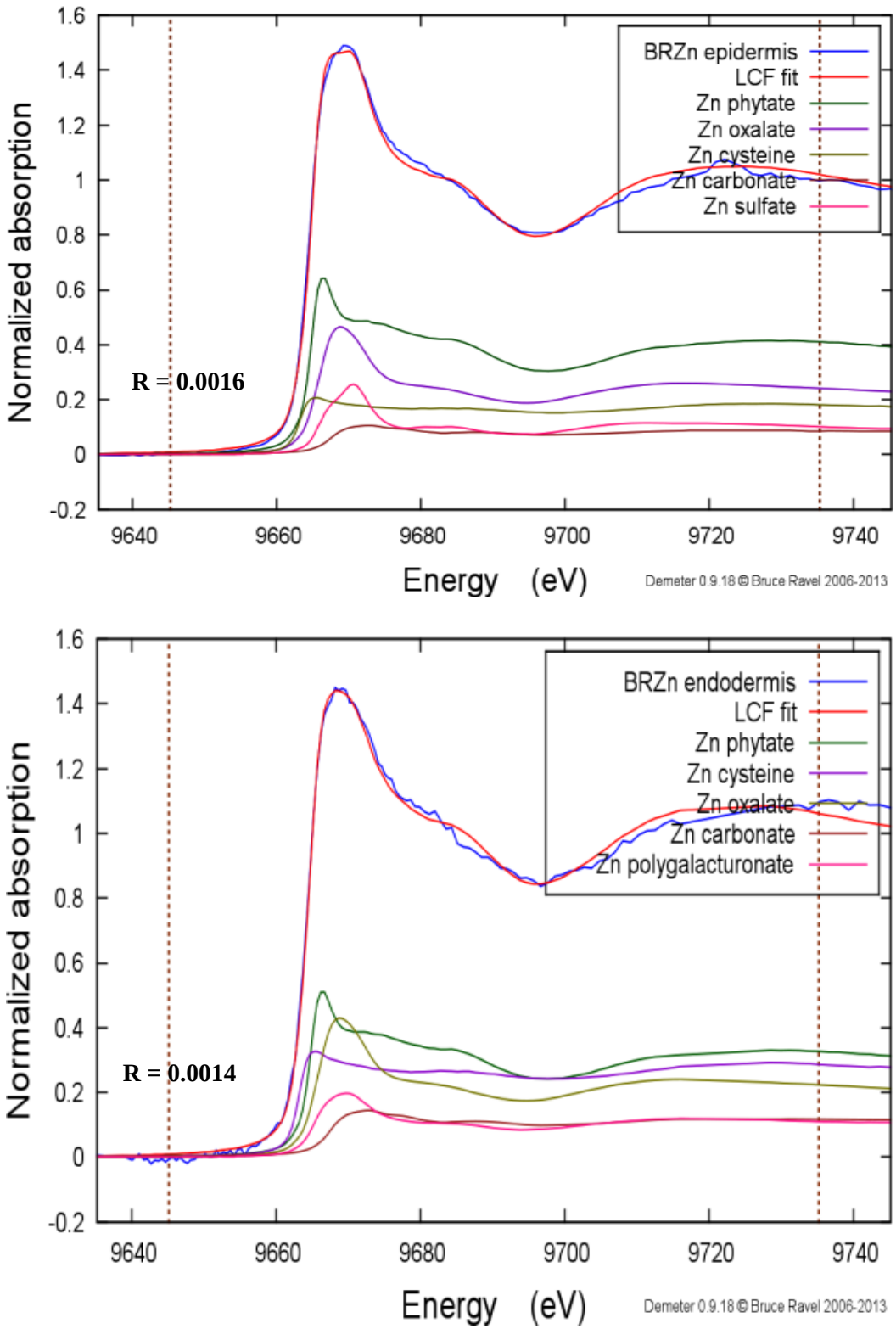



**Figure 7.10 (c): Zn K-edge XANES fitting, R-factors and Zn-compound compositions for epidermis and endodermis of root inoculated with *R. leguminosarum* (BRZn). *R* = Σi (experimental-fit)$^2$ / Σi (experimental)$^2$. The lower the *R* value the better the fit.**

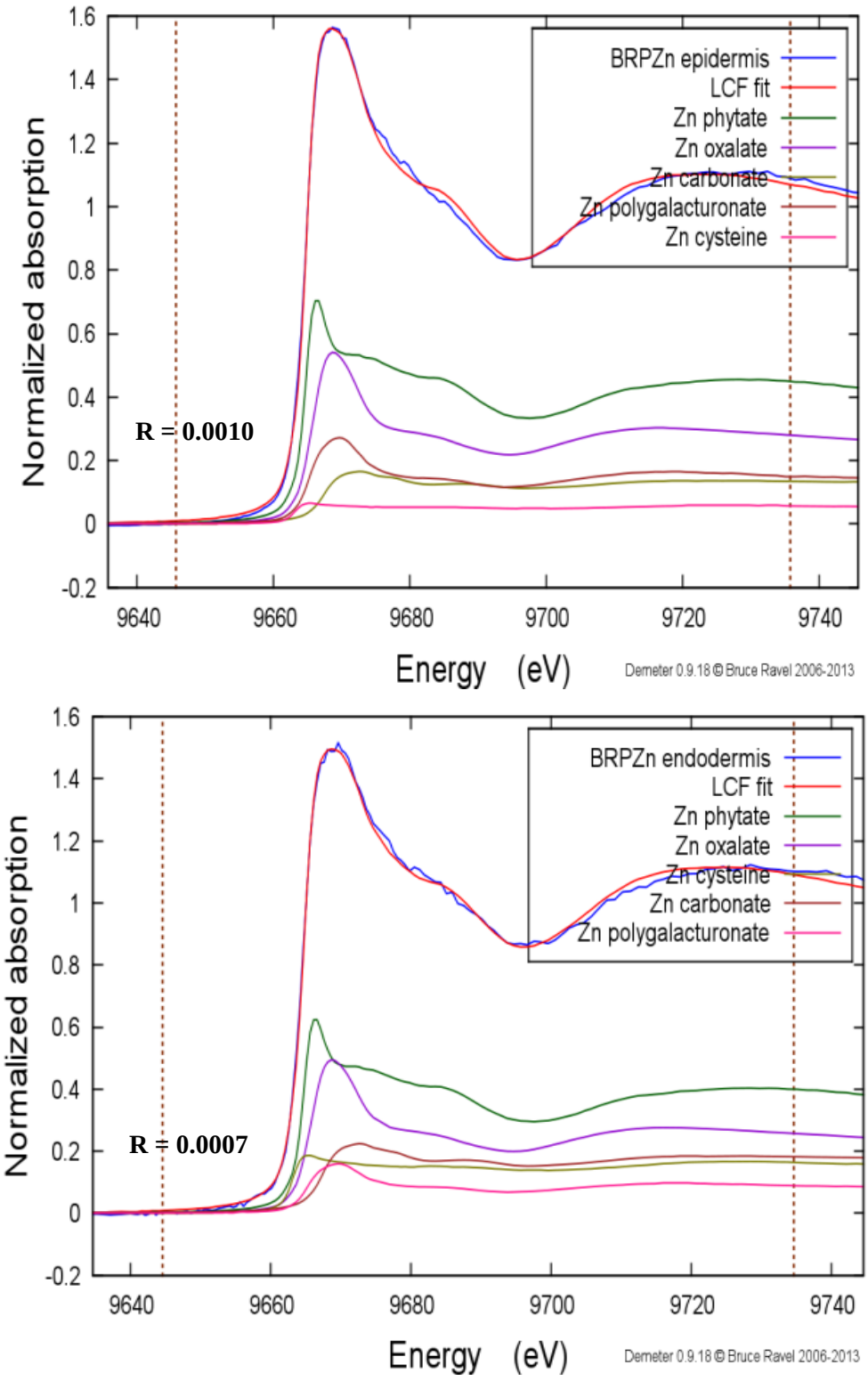



**Figure 7.10(d): Zn K-edge XANES fittings, R-factors and Zn-compound compositions for epidermis and endodermis of root inoculated with a combination of *R. leguminosarum* and *P. brassicacearum* (BRPZn). *R* = Σi (experimental-fit)$^2$ / Σi (experimental)$^2$. The lower the *R* value the better the fit.**

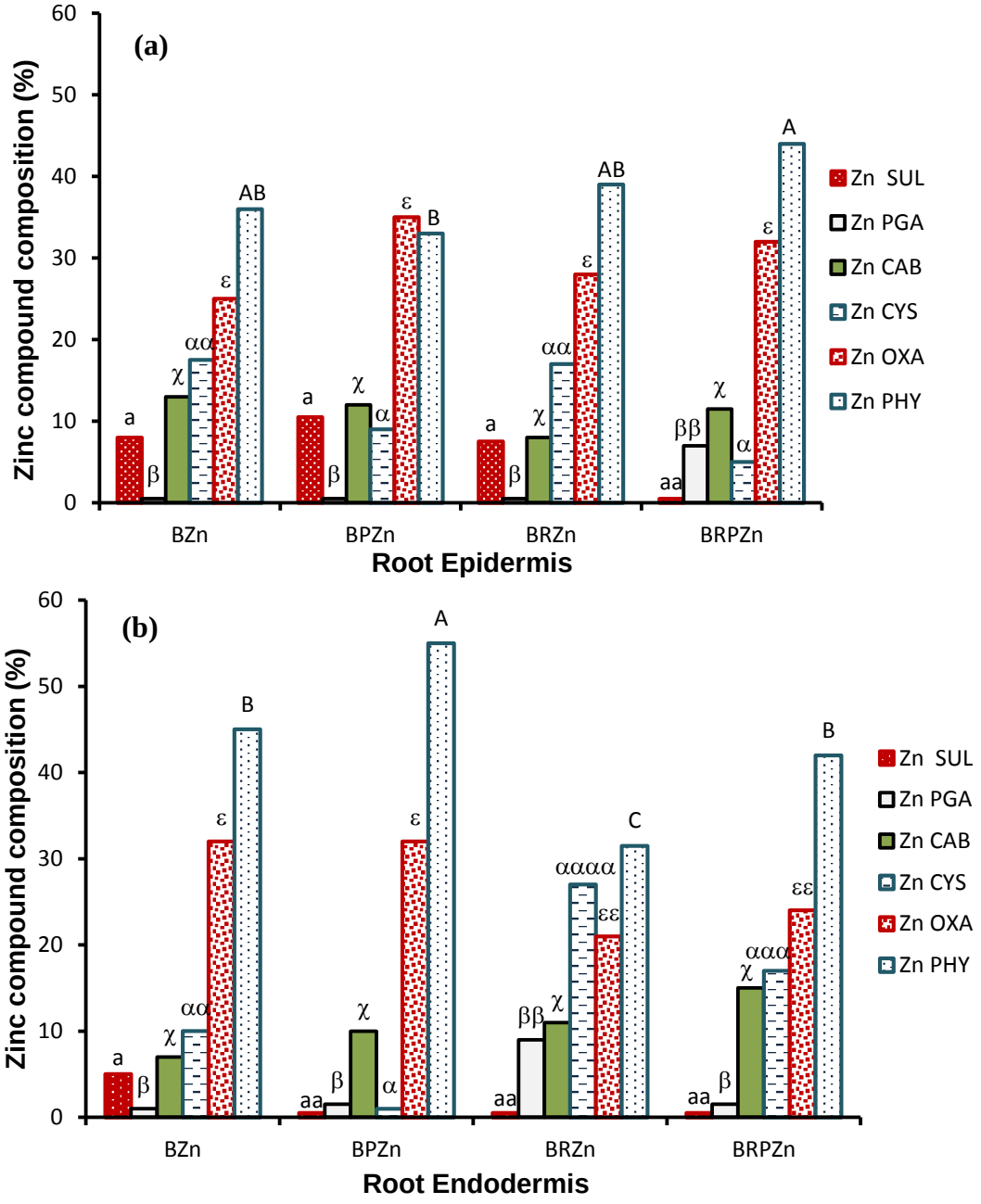



**Figure 7.11: Zn compound compositions (%) in the root (a) epidermis and (b) endodermis. Zn SUL – Zn sulphate, Zn PGA – Zn polygalacturonic acid, Zn CAB- Zn carbonate, Zn CYS - Zn cysteine, Zn OXA – Zn oxalate and Zn PHY- Zn phytate. Bar are means of Zn compound compositions from 6 and 3 Zn XANES collected from the epidermis and endodermis respectively of two replicate root samples per treatment. Different symbols show significant differences ($p<0.05$) between treatments.**

A comparison of Zn speciation at the epidermis and endodermis of un-inoculated *Brassica juncea* roots shows no differences in the types of Zn compounds present at these regions, however there were changes in Zn speciation between the root epidermis and endodermis of bacterial inoculated plants. In roots inoculated with *P. brassicacearum* (BPZn) and *R. leguminosarum* (BRZn), Zn sulphate appeared to be sequestrated only at the root epidermis with the endodermis free of the Zn sulphate. Moreover, by comparing Zn compound compositions at the epidermis among all the treatments, there were statistically significant differences in the presence of Zn sulphate and Zn polygalacturonic acid in the BRPZn treatments compared to the others. Zn sulphate appeared to be completely removed or transformed to Zn polygalacturonic acid only in BRPZn root epidermis. Moreover, the proportion of Zn present as Zn phytate in the root epidermis was significantly higher in the BRPZn treatment compared to other treatments.

Furthermore, among all the principal Zn species in the root endodermis and epidermis, the only species with no significant difference in % composition between the treatments is Zn carbonate composition. The % of Zn present as Zn sulphate was significantly higher in BZn than the bacteria inoculated treatments. This result is consistent with the result of Zn speciation in the root of *B. juncea* at 5 weeks after planting in Zn contaminated soil (see Chapters 5 and 6). The proportion of Zn oxalate in the root endodermis in BZn and BPZn treatments were significantly higher than the BRZn and the BRPZn treatments. The highest proportions of Zn phytate and Zn cysteine were observed in the endodermis of the BPZn and BRZn treatments respectively. Apart from the statistically significant lower proportions of Zn oxalate

in the BRZn and BRPZn, the proportions of Zn cysteine in the two treatments were higher than the BZn and BPZn.

## 7.4. Discussion

The major findings from this study are as follows:

(i) Inoculation of *B. juncea* seeds with PGPB improved seed germination but only under Zn contamination

(ii) *R. leguminosarum* and its combination with *P. brassicacearum* significantly promoted root growth under Zn contamination

(iii) Zn accumulation was higher at the epidermis of roots where bacteria were localised

(iv) There were significant differences in Zn speciation between the epidermis and endodermis of PGPB inoculated and un-inoculated roots exposed to Zn contamination

### 7.4.1. Effects of PGPB on seed germination under Zn contamination

The seed coat has been reported to provide some level of protection to the seed embryo from metal stress (Wierzbicka and Obidzińska, 1998, Kranner and Colville, 2011). Under prolonged exposure to metal contamination however, the seed coat becomes more permeable, ruptures and the radicle becomes visible (Li et al., 2005b). The emerging radicle is then exposed to the direct effects of toxic metal contamination (observed within 2-3 days in this experiment), leading to potential termination of radicle development and growth (Munzuroglu and Geckil, 2002, Li et al., 2005b). It is therefore likely that the seed coat of the *B. juncea* seeds protected the embryo from metal toxicity and the hindrance of radicle development (< 1 mm

length) in the BZn treatments was due to the direct exposure of the radicle to Zn contamination.

Although there have been no previous studies on the effects of Zn contamination on *B. juncea* seeds exposed to Zn 400 mg $kg^{-1}$ for up to 14 days, some studies have reported no significant effects of Zn contamination on seed germination in other plant species (Ozdener and Kutbay, 2009, Marichali et al., 2014) while results similar to the one observed in this experiment have also been reported (Márquez-García et al., 2013, Wang et al., 2011).

Apart from the protective effects of the seed coat, chelation of metals within seeds and the induction of antioxidant defences during germination has been suggested as a possible mechanism through which seeds survive metal toxicity and germinate into seedlings in metal contaminated environments (Kranner and Colville, 2011). Phytochelatins and metallothioneins are metal-binding peptides that are known to induce metal tolerance in plants and are mostly observed in plant roots and leaves (Cobbett and Goldsbrough, 2002, Kranner and Colville, 2011). These metal chelates however, have also been observed to be synthesised in seeds and reported to enhance seed tolerance to toxic concentrations of metals (Li-Chan et al., 2002, Brkljačić et al., 2004, Kranner and Colville, 2011).

It has been demonstrated (Chapter 5) that *R. leguminosarum* and its combination with *P. brassicacearum* induced the secretion of chelates like phytate and cysteine in plant roots exposed to Zn contamination. It is therefore likely that the bacteria strains induced the secretion of these chelates in the seeds or germinating radicle, and thus helped ensure optimum seed germination under Zn. The seed germination percentage under Zn contamination in BRZn and BRPZn treatments was not significantly

different from germination of seeds in un-contaminated media (Bo), an indication that the Zn toxicity to seeds was ameliorated in these treatments.

### 7.4.2. Mechanisms of *B. juncea* growth promotion and enhanced Zn bioaccumulation by PGPB under Zn contamination

The mechanism of plant growth promotion by PGPB has been extensively described by many authors to be based on bacterial improvement of plant nutrition through nitrogen fixation, phosphate release and siderophores secretions (Khan et al., 2009, Rodríguez and Fraga, 1999, Compant et al., 2010). The apparent absence of any significant improvement in plant growth in uncontaminated media, however, suggests that plant nutrients were not a limiting factor and the possibility of plant growth promotion through improvement in plant nutrition was therefore unlikely in this experiment. The better tolerance to Zn contamination observed in roots inoculated with bacteria may therefore be due to the ability of the PGPB to attenuate Zn toxicity, either through changes in Zn speciation or through sub-cellular Zn compartmentalisation in the plant root, or through the combination of the two mechanisms.

Moreover, from the results of CLSM images analysis, the presence of Zn in the root did not prevent bacterial colonisation of the root epidermis while the endodermis was relatively free of bacteria cells. The ability of the bacterial strains to survive Zn contamination has been previously described and discussed in Chapter 4 and by other authors, who have identified possible mechanisms for bacterial survival under metal toxicity as efflux of metal ions outside the bacterial cell wall, metallothioneins sequestration and reduction of metal ions to less toxic forms (Choudhury and

Srivastava, 2001, Miller et al., 2009, Trevors et al., 1985). Results from this study however further add to knowledge about the nature of bacterial colonisation between the two bacterial strains.

*R. leguminosarum* is a rhizospheric bacterium that mainly resides at the rhizosphere (a soil-root interphase) (Reeve et al., 2010) and its penetration ability into the root (especially in plants like *B. juncea* that do not have nodules) is limited compared to *P. brassicacearum* which was isolated from the rhizosplane (a sphere deeper into the root than the rhizosphere) and has been demonstrated to exhibit endophytic properties in this research and in other studies (Long et al., 2008). In this study, when Zn accumulation at the epidermis of un-inoculated root appeared to be low, the two PGPB significantly enhanced Zn bioaccumulation and clearly induced different levels of Zn sequestration at the root epidermis due to the nature of their plant root colonisation.

The significantly higher Zn tolerance index in roots under *R. leguminosarum* inoculation over *P. brassicacearum* may be due to the isolation of excessive Zn from the endodermis and subsequent Zn deposition at the epidermis where the bacteria was predominantly localised. On the other hand, the reduced root tolerance index observed in the BPZn treatment may be due to enhanced Zn deposition at the epidermis and also significant Zn diffusion into the endodermis due to the endophytic root colonisation characteristics of *P. brassicacearum*. The BRPZn treatments appeared to have benefited from the combination of the rhizospheric and

endophytic root colonisation nature of both PGPB because the highest amount of Zn accumulation at the root epidermis occurred in this treatment.

Results from Zn speciation analysis at the epidermis and endodermis of the treatments suggest enhanced Zn sequestration at the root epidermis as not the only means through which the PGPB conferred Zn tolerance to the plant root, and that change in metal speciation may also play important role.

Since Zn sulphate solution was the form of Zn contamination used in this experiment it was therefore not surprising that Zn sulphate was detected as a principal Zn component both at the root epidermis and endodermis of un-inoculated seedlings. The stability constant of $ZnSO_4$ in pH conditions similar to the one in plants has been estimated to be log $K$ = 2.38 (Wang et al., 1992). This suggests that the compound readily dissociates to yield ionic $Zn^{2+}$ in the plant system. This form of Zn complex therefore offers no protection to the roots and the presence Zn as Zn sulphate at both root epidermis and endodermis may contribute to the reduced root growth observed in the BZn treatment in this experiment.

Moreover, complexation of Zn with carbonates and carboxylic acids like oxalates and polygalacturonic acids has been observed and reported to be an important response mechanism to metal toxicity in plants under high Zn concentrations (Dalvi and Bhalerao, 2013, Jones, 1998). The root cell wall and the vacuole contain carbonaceous materials that can react with excessive Zn ion concentration to reduce its toxic effects (Hall, 2002, Wang et al., 1992). Complexation of Zn with polygalacturonic acids ($C_{12}H_{14}O_{12}Zn.4H_2O$) for example, has been reported to

enhance tolerance to Zn toxicity in some plants (Cataldo et al., 2012, Dalvi and Bhalerao, 2013). Although Zn polygalacturonic acid appeared not to be synthesized in the roots of un-inoculated seedlings in this study, the epidermis of the BRPZn treatment appeared to store Zn as Zn polygalacturonic acid instead of Zn sulphate and there was significant higher Zn polygalacturonic acid in the endodermis of the BRZn treatment. It is worthy of note that better root tolerance to Zn toxicity was observed in these treatments.

Furthermore, the proportion of Zn occurring as Zn oxalate in both root endodermis and endodermis among all the treatments was significantly higher than any other organic acids, an indication of higher stability of the complex in the seedling roots. This phenomenon has been observed in other hyperaccumulating plants (Sarret et al., 2002) and the reaction of Zn with oxalate has been reported to form a more stable Zn oxalate complex ($ZnC_2O_4$) with a stability constant log $K = 4.68$ (Sillén and Martell, 1964). However, high concentrations of this soluble acidic Zn oxalate complex in metal resistant plants have been linked with enhanced Zn toxicity to plants (Mathys, 1977). This may also explain the reduced root growth observed in the BZn and BPZn treatments since the proportion of Zn oxalate in their endodermis is significantly higher compared to the endodermis of BRZn and BRPZn treatments both of which exhibited better root growth.

In addition to the widely reported complexation of carboxylic acids with Zn, amino acids are also major cellular ligands known to complex with Zn in plants (Zeng et al., 2011, Salt et al., 1999). Metalothioneins are low-molecular-weight, cysteine-rich

proteins which provide thiols for metal chelation (Steffens, 1990, Sinclair and Krämer, 2012). A Zn (II)-L-cysteine complex with a stability constant log $K$ = 9.80 (Marešová et al., 2012, Serap Karadert, 2014) offers a higher resistance to protolytic degradation compared to the Zn organic acid complexes and therefore helps reduce the cellular bioavailability of Zn (II) (Kelly et al., 2002, Cobbett and Goldsbrough, 2002). Cysteine has been identified to be important in metal homeostasis and detoxification and tolerance to elevated concentrations of Zn in many plants has been associated to Zn – cysteine chelation (Zeng et al., 2011, Kelly et al., 2002). Moreover, Zn binds relatively strongly to hard ligands (O and N) but weakly to soft (S) ligands (Garmer and Gresh, 1994, Kinraide, 2009). Cysteine contains the S ligand and it is therefore regarded not to be involved in enhancing Zn accumulation but only in conferring Zn toxicity tolerance through Zn chelation in plants (Kopittke et al., 2011). The evidence of Zn cysteine in un-inoculated *B. juncea* seedlings is a reflection of the inherent ability of the plant to reduce the toxic effects of Zn in the root through chelation. However, more of the Zn cysteine (17%) was formed at the root epidermis compared to in the endodermis (10%) where the Zn sensitive organelles are located. Having more of Zn cysteine in the inner part of the root would have offered reduced Zn bioavailability to sensitive plant organelles and better overall root tolerance to Zn contamination. While *P. brassicacearum* appeared not to be able to stimulate cysteine production in the seedlings root, *R. leguminosarum* significantly enhanced chelation of Zn by cysteine in the root endodermis.

In other research that studied the gene sequence of *Rhizobium leguminosarum*, the bacterial strain was revealed to contain acetyl transferases lacA and cysE genes (Downie, 1989). The biosynthesis of cysteine from sulphate in plants has also been

demonstrated to be catalysed by acetyl transferase enzymes (Bogdanova and Hell, 1997). Although *R. leguminosarum* was imaged to be localized mainly at the plant epidermis, it is likely that the bacteria strains secrete enzymes that facilitated cysteine synthesis from sulphate (probably obtained from the Zn sulphate solution used as the source of Zn contamination) in the root endodermis. The better plant growth observed in the BRZn and BRPZn treatments is therefore attributed to the significant increase in the storage of Zn as Zn cysteine in the endodermis from 10 % (in the BZn) to 27% in BRZn and 17% in BRPZn treatments.

Furthermore, Phytate (myo-inositol hexakis (dihydrogen phosphate), $C_6H_{18}O_{24}P_6$; IP6) poses strong negatively charged phosphate groups and reacts with Zn to form insoluble Zn phytate complex (Crea et al., 2008, Kopittke et al., 2011, Marešová et al., 2012). For example, in a colorimetric and titrimetric study of the reaction of phytic acid with Zn at pH < 6 and 25$^{o}$C, an insoluble Zn phytate complex with a stability constant log $K$ = 30.4 was formed (Martin and Evans, 1986). Immobilisation of Zn as stable Zn phytate in plant organs has been observed in both hyperaccumulating and non-hyperaccumulating plants and it is increasingly being recognised as one of the mechanisms for enhanced metal accumulation and tolerance in plant roots (Van Steveninck et al., 1990, Kopittke et al., 2011).

Although globular deposits of Zn phytate are mostly observed in the endodermis of dicotyledonous plants and in the pericycle of monocotyledonous plants, it has also been observed to be deposited in the stele and inner cortex after prolonged exposure to toxic levels of Zn (Van Steveninck et al., 1994). Sequestration of Zn as Zn phytate in the root has also been reported to restrict root to shoot Zn translocation (Van

Steveninck et al., 1993). In this experiment, apart from the epidermis of the BPZn treatment in which the proportion of Zn oxalates slightly exceeds that of Zn phytate, Zn phytate accounted for the highest proportion of Zn storage in both the epidermis and endodermis of the treatments. This significant immobilization of accumulated Zn as Zn phytate in the roots may be responsible for the lack of Zn toxicity observed in the shoots of seedling exposed to Zn contamination when compared to shoot of plants in un-contaminated media.

The storage of Zn as Zn phytate was significantly higher in the endodermis of all the treatments than the epidermis, indicating that the vacuole, the organelles responsible for storing metals in root endodermis, may be involved in the sequestration of Zn in *B. juncea* (Kopittke et al., 2011). Moreover, the greatest significant differences in the proportions of Zn phytate among the treatments were also observed in the endodermis, an indication that the bacteria influence vacuolar Zn sequestration, with *P brassicacearum* appearing to have greater influence than the *Rhizobium leguminosarum*. This may be due to the ability of *Pseudomonas brassicacearum* strain to colonise the internal plant structure.

However, in this study, sequestration of Zn as Zn phytate does not correlate with better root tolerance as reported in other studies (Terzano et al., 2008, Kopittke et al., 2011). The BPZn treatments that had the highest proportions of Zn occurring as Zn phytate in the plant endodermis exhibited lower root tolerance to Zn contamination compared to the BRZn and the BRPZn treatments. It is likely that the high amount of Zn phytate in the endodermis is not enough to protect the roots from being susceptible to Zn in the form of oxalate which is also significantly higher in the

endodermis of this treatment than the other bacterial inoculated treatment. The significantly higher Zn accumulation in the BRPZn seedlings and the better root tolerance to Zn toxicity may be traced to the storage of Zn predominantly as Zn phytate at the epidermis and endodermis, coupled with the significant chelation of Zn in the form of Zn cysteine.

In our study where Zn speciation was determined in mature (5 weeks) roots of *B. juncea* plant (Chapter 5), Zn in the BZn treatments was predominately stored as Zn sulphate and Zn oxalate while Zn polygalacturonic acid, phytate cysteine and carbonate were the main forms of Zn in the treatments inoculated with bacteria. When compared to the multiple Zn components (especially in BZn) observed in this study, it is likely that Zn speciation changes under continuous exposure to Zn contamination or with respect to the age of the plant. Nevertheless, *Rhizobium leguminosarum* and its combination with *P. brassicacearum* significantly promoted plant growth and Zn accumulation under Zn contamination by attenuating metal toxicity while enhancing Zn bioaccumulation, at every stage of plant growth.

### 7.5. Conclusion

In conclusion, seeds of un-inoculated *B. juncea* plants were susceptible to Zn toxicity and growth of seedling roots was significantly hindered under Zn contamination. Although there was a high level of Zn sequestration as Zn phytate in the root endodermis of BZn treatment, there were no difference in the species of Zn at both the epidermis and endodermis of the plant root. This is contrary to the hypothesis that there will be changes in the species of Zn between the epidermis and endodermis

of un-inoculated plant root due to differences between the nature and properties of the cell wall (at the epidermis) and root vacuole (at the endodermis).

Furthermore, although inoculation with *P. brassicacearum* slightly improved seed germination under Zn, it had no significant effects on Zn tolerance in roots. The bacterial strains however colonise the root and enhance Zn accumulation both at the root epidermis and endodermis. Increased storage of Zn as Zn sulphate and Zn oxalate at the expense of Zn cysteine may be responsible for root susceptibility to the Zn toxicity observed. On the other hand, *R. leguminosarum* significantly enhanced seed germination and promoted root growth under Zn contamination. It colonised the root epidermis and significantly enhanced accumulation of Zn at the root epidermis thus isolating excessive Zn from the endodermis where Zn sensitive organelles are located. Coupled with induced Zn compartmentalization of Zn at the endodermis, *R. leguminosarum* enhanced the secretion of Zn cysteine in the root endodermis. A combination of the two bacteria resulted in full recovery of the seeds and seedlings from metal toxicity compared to un-inoculated control; significantly enhanced accumulation of Zn at the epidermis compared to the other treatments and attenuated toxicity by Zn-cysteine and phytate chelation.

As hypothesised, both bacteria strains enhanced Zn localisation at the epidermis where they were mostly colonised but also influenced changes in Zn speciation in the endodermis. Enhanced Zn compartmentalization at the root epidermis and bacterial mediated reduction in Zn toxicity through changes in Zn speciation may therefore be key complimentary mechanisms of plant growth promotion and enhanced Zn accumulation in plants induced by plant growth promoting bacteria.

Chapter 8

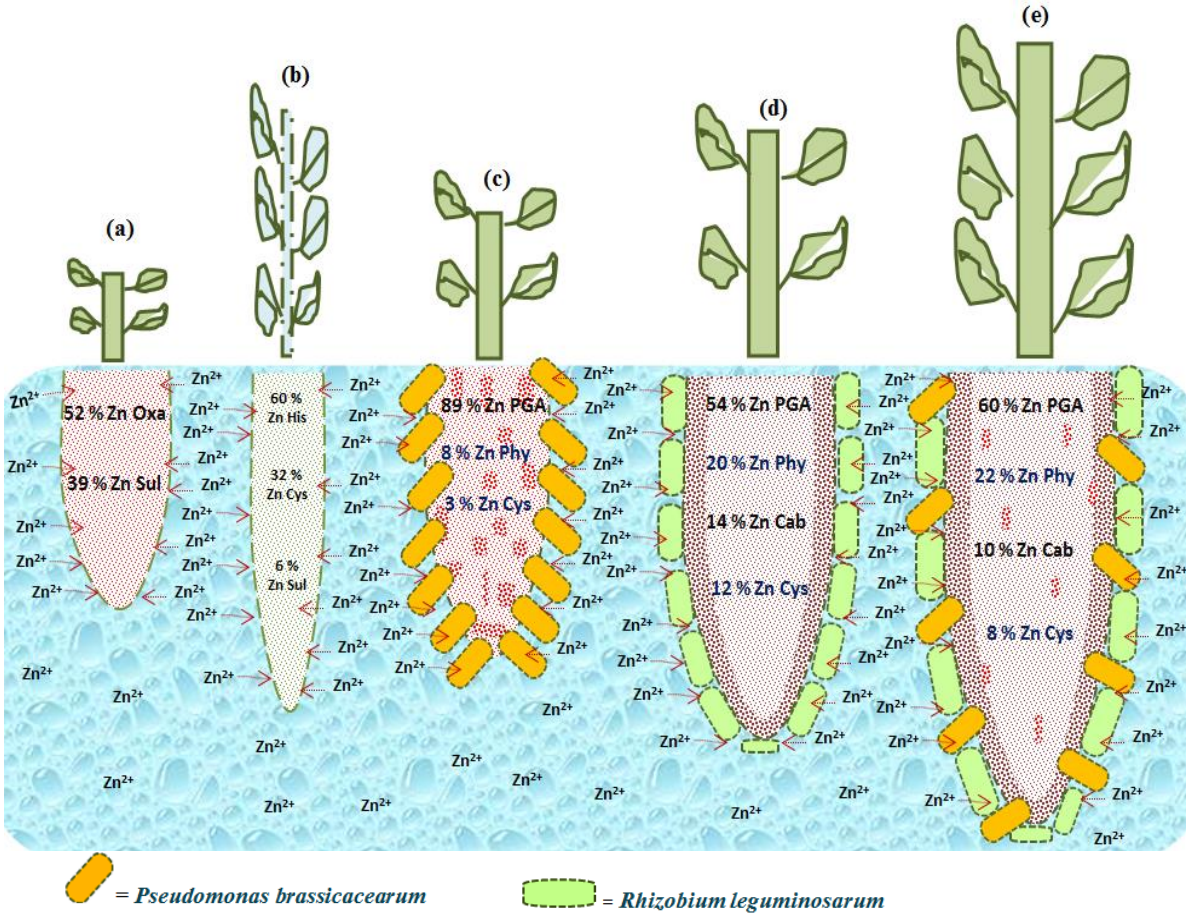
# 8. Summary of research results, prospects and future studies

## 8.1. Introduction

The goal of this PhD research is to evaluate the use of plant growth promoting bacteria (PGPB) and a leguminous plant to enhance the growth and metal remediating potential of a phytoremediating plant in metal contaminated environments. The research also specifically aims to use state-of-the-art techniques to unravel the mechanism(s) of better plant growth in the face of higher metal bioaccumulation in a phytoremediating plant inoculated with PGPB and mixed planted with a leguminous plant.

*Brassica juncea*, a well-known accumulator of many metal contaminants was selected as the phytoremediating plant. *Pseudomonas brassicacearum* isolated from a Brassica plant and *Rhizobium leguminosarum* isolated from a clover plant were used as the PGPB. The leguminous plant *Vicia sativa* was established to be tolerant to high soil Zn contamination and was evaluated for its ability to promote the growth of the *B. juncea* plant in a mixed planting system. Growth experiments were conducted under glasshouse conditions in soils contaminated with Zn 600 and 400 mg $kg^{-1}$ and under sterile conditions in media contaminated with 400 mg $kg^{-1}$ Zn. The results of this research are summarised in Figure 8.1 and discussed below.

## 8.2. Summary of results




Zn Oxa - Zn oxalate, Zn sul – Zn sulphate, Zn PGA – Zn polygalacturonic acid, Zn Cab –Zn carbonate, Zn Phy – Zn phytate, Zn His - Zn histidine, Zn Cys – Zn cysteine

(a) *Brassica juncea* plant root. Stunted plant growth and low Zn bioaccumulation attributed to root storage of Zn as Zn oxalate and sulphate

(b) *Vicia sativa* plant root. Zn hypertolerance but lower Zn accumulation (compared to (a)) in the leguminous plant was attributed to root storage of Zn as Zn cysteine and histidine

(c) *B. juncea* plant root inoculated with endophytic *P. brassicacearum*. Inoculation with the PGPB enhanced Zn bioaccumulation. The dispersed Zn hot spots in the root and storage of Zn mainly as Zn polygalacturonic acid may be responsible for reduced plant growth

(d) *B. juncea* plant root inoculated with rhizospheric *R. leguminosarum*. Inoculation with the PGPB significantly enhanced Zn bioaccumulation more than (c). The sub-cellular compartmentalisation of Zn at the root epidermis where the PGPB colonised and storage of Zn as Zn phytate and Zn cysteine are likely responsible for the high Zn bioaccumulation

(e) *B. juncea* inoculated with *P. brassicacearum* and *R. leguminosarum*. Dual bacterial inoculation conferred multiple growth effects under Zn contamination. Plant growth and Zn bioaccumulation was significantly higher than in all other treatment

**Figure 8.1: A summary of thesis results depicting plant growth and nature of Zn sequestration and speciation in un-inoculated and PGPB inoculated treatments (plant roots (a)-(e)) studied at 5 weeks after planting in soil contaminated with Zn 400 mg $kg^{-1}$. Better plant growth and higher Zn bioaccumulation in (d) and (e) was attributed to the storage of Zn as Zn cysteine and phytate in the root of the plants**

The growth of un-inoculated *B. juncea* was significantly stunted under Zn contamination. This was attributed to the storage of zinc in the form of Zn sulphate and oxalate in the plant's root (Figure 8.1a). It was hypothesised that inoculation of the *B. juncea* plant with a native PGPB (*P. brassicacearum)* should facilitate root colonisation under Zn contamination and lead to better growth and Zn bioaccumulation compared to un-inoculated *B. juncea* plants. However, plant growth and Zn bioaccumulation in *B. juncea* was only slightly promoted under inoculation with *P. brassicacearum* in the two experiments conducted in a glasshouse and in the laboratory based plant growth promotion assessment under sterile experimental conditions. This was traced to the endophytic root colonisation nature of *P. brassicacearum* and the inability of plants inoculated with the bacterial strain to prevent the accumulation of toxic forms of zinc in the root endodermis where Zn sensitive organelles are located (Figure 8.1c).

On the other hand, *R. leguminosarum*, the bacterial strain that is non-native to Brassica plants significantly promoted plant growth and enhanced Zn bioaccumulation in *B. juncea*. This was traced to the ability of the bacterial strain to significantly colonise the root epidermis, with epidermal bacterial colonisation leading to enhanced Zn sequestration at the root epidermis. Coupled with enhanced epidermal Zn sequestration, *R. leguminosarum* significantly induced Zn immobilisation through the formation of Zn phytochelatins and metallothioneins (as Zn cysteine and phytate) both in the roots of *B. juncea* seedlings and mature plants (Figure 8.1d).

The use of a metal tolerant bacterial strain that is native to a phytoremediating plant to enhance the growth and metal accumulation potential of the plant is being strongly advocated (Idris et al., 2004, Sheng et al., 2008, Luo et al., 2011). Results from this research however suggest that the nature of bacteria-root colonisation in metal contaminated environments, and type(s) of metal species induced by bacteria inoculation in a phytoremediating plant, are key parameters to be considered when selecting PGPB for enhancing plant growth and metal bioaccumulation in contaminated environments.

As hypothesised, dual inoculation of *B. juncea* with both *P. brassicacearum* and *R. leguminosarum* promoted plant growth better than single bacterial inoculation and also enhanced Zn bioaccumulation the most under Zn contamination (Figure 8.1e). It is apparent that the plant benefited from the nature of root colonisation as well as changes in Zn speciation induced by the two bacterial strains. Bacteria are being genetically modified to possess multiple beneficial plant growth promoting traits (Weyens et al., 2013, Qiu et al., 2014). This research however recommends the use of two or more natural bacteria strains, with synergetic growth promoting and toxicity attenuation effects on a hyperaccumulating plant, as a more sustainable method for remediation of soils contaminated with metals.

Furthermore, although the use of legumes to promote the growth of crops in nutrient deficient soils is well known (Bedoussac et al., 2014, Sturludóttir et al., 2014), this research is the first to report the use of a non-metal accumulating but metal tolerant leguminous plant (*Vicia sativa*) to promote growth and metal remediation in a less tolerant but metal remediating plant (*B. juncea*), in a process termed – legume-

assisted phytoremediation. Through the use of synchrotron μ-XRF imaging combined with μ-XANES analysis it was discovered that the leguminous *V. sativa* plant synthesised histidine and cysteine (Figure 8.1b) and the synthesis of these metal chelates was attributed to the better growth of *B. juncea* and enhanced Zn phytoremediation observed in the mixed planting treatment. The legume assisted phytoremediation system also offers sustainable alternatives to the use of chemical chelates like EDTA which has been reported to be accompanied by potential secondary metal contamination (Wu et al., 2004, Nowack et al., 2006, Leštan et al., 2008).

### 8.3. Future works

Despite the achievements of this research, more work is still needed before microbial assisted phytoremediation or legume assisted phytoremediation can be implemented as a successful remediation technology. There are also other scientific questions to which the research results can contribute. These are highlighted as follows:

(1) **An evaluation of changes in Zn accumulation and speciation by PGPB and legumes in plants exposed to real-life contaminated soils**

This study utilised fertile soil that was sterilised and artificially contaminated with soluble Zn in order to focus on understanding the mechanisms of plant growth promotion with regards to understanding Zn speciation in plant tissues. As a consequence, the results may be somewhat biased by the relative higher Zn bioavailability compared to real-life contaminated soils (Chlopecka and Adriano,

1996, Smit and Van Gestel, 1996). It is therefore necessary to expand these studies to using soils obtained from various Zn contaminated sites.

Moreover, it is not clear the type of impacts the inoculated bacteria strains will have on the biology of the contaminated soil system. It is also possible that the microorganisms that are already present in the contaminated soil may influence the plant growth promoting ability of the bacterial strains used in this research or induce another types of growth promoting mechanisms under Zn contamination apart from the one reported in this study. It is therefore also important to conduct experiments similar to the ones reported in this study with un-sterilised real-life contaminated soil.

Furthermore, it is also likely that the legume – metal phytoremediator mixed planting system may have other effects (apart from growth promotion and enhanced Zn accumulation) on the biology and general nutrient dynamics in the contaminated soil system. An assessment of the impacts of PGPB inoculation and legume mixed planting on biological, physical and chemical properties of natural contaminated soils will therefore contribute to knowledge of ecological impacts of microbial and legume assisted phytoremediation in Zn contaminated soils.

(2) **Mechanism (s) of plant growth promotion and enhanced remediation by PGPB and legumes in soil contaminated by multiple metal contaminants**

This research focused only on the biochemistry of Zn. However, the majority of metal contaminated soils are contaminated with more than one metal contaminant and metals differ in their level of toxicity (Lombi et al., 2001, do Nascimento et al., 2006). For example, will the presence of Cd affect the nature of Zn toxicity, bioaccumulation and speciation in plant roots? In other word, if bacteria induce the

secretion of phytate and cysteine in plant under multiple metal contamination will there be a competition among the metals for ligands? Will the level of bacterial survival in a system contaminated with Cr (IV), Cd and Zn be the same as reported for Zn? Will the nature of bacterial colonisation of roots and subcellular metal sequestration differ in plants under single metal contamination and in plants exposed to multiple metal contaminants? In order to answer these questions, it is important to study the mechanisms of plant growth promotion and enhanced metals bioaccumulation by PGPB and legumes in experimental conditions contaminated with a mixture of metals and compare the results with the one reported in this study.

(3) **What is the mechanism behind phytate synthesis in *B. juncea* under Zn contamination?**

Better plant growth and significantly higher Zn accumulation were consistently observed in treatments with significantly higher proportion of phytate. But where do the phytate comes from? The structure of phytic acid is complex (Figure 8.2c) and because phosphorous (P) is a key component of phytic acid the simple answer would have been – the phytate comes from the soil since a fertile soil with high concentrations of available P was used in the experiment (Figure 8.2d).

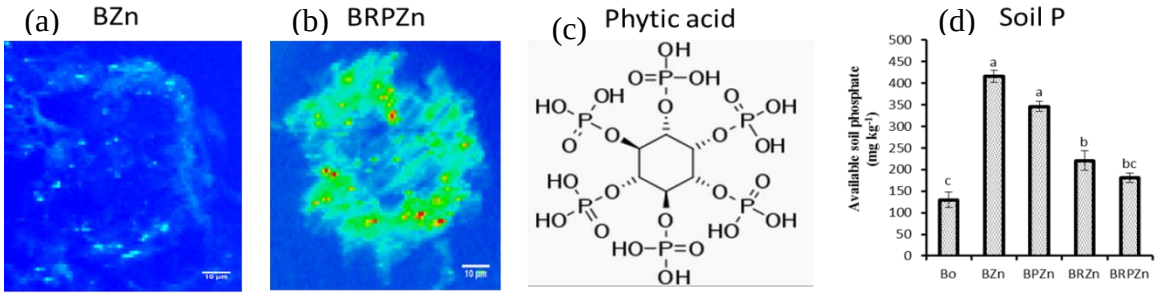



**Figure 8.2: Zn accumulation in *B. juncea* (a) un-inoculated and inoculated with PGPB where warm colours represent higher Zn concentrations (b) the structure of phytic acid, appears to sequester Zn in PGPB inoculated plant (d) reduction in soil bioavailable phosphate at 5 weeks after planting enhanced by PGPB inoculation under Zn contamination**

However, there was complete absence of phytate in un-inoculated plants established in soil with high concentration of available P (see Figure 8.1a) an indication that the picture of phytate synthesis is more complex. Moreover, in analysis of seedlings established in sterile wiper paper, phytate was present in the roots of un-inoculated plants as well as in plants inoculated with PGPB (see Figure 7.1). Phytate is known to be the form in which plants store phosphate, particularly in seeds (Bentsink et al., 2003, Dorsch et al., 2003). It is therefore likely that phytates was liberated from the seeds to enhance seedlings growth and metal bioaccumulation and the amount of phytates gradually disintegrates as the plant continues to grow under metal contamination. This may explain why phytate was detected in the roots of 14 days old seedlings of un-inoculated *B. juncea* but not in mature (5 weeks) plant roots under Zn contamination. Furthermore, another critical question to ask is; how was phytate secretion from the seeds triggered – by metal toxicity or bacterial inoculation or both? Another important question to add to that is; how was phytate composition maintained in the roots of plants inoculated with *R. leguminosarum* and combination of *R. leguminosarum* and *P. brassicacearum* from seedling germination stage to the stage of mature plant root (see figure 7.1 and 8.1)?

Many human and animal trace metal nutritional requirements are obtained through grains and seeds which they accumulate from the soil (Ali et al., 2010). Soils deficient in such trace metals or where the metal exists in non-bioavailable forms lead to these nutritional deficiencies. A successful identification of the mechanism behind enhanced metal accumulation by PGPB as stable metal-phytate chelates in edible plant parts will go a long way in providing solutions to ameliorating the

problem of nutritional deficiency (e.g. zinc and selenium) especially in sub-Saharan Africa countries (Hurst et al., 2013).

It is therefore important to use the knowledge acquired through this study to develop experimental methods and explore analytical methods for investigating the mechanisms of phytic acid synthesis and other ligands (such as cysteine) that have been identified. As examples, the following approaches should be explored:

(i) Studies suggest that seeds release most of their phytic acid during germination as a mechanism for supplying the seedlings with phosphorus. Hence the question of whether bacteria enhance the release of phytic acid at germination stage can be addressed by extraction and quantification of phytic acid during germination experiments. Analysis of phytate in seeds and seedlings inoculated with bacteria in the absence of metal contamination may also help to know if phytic acid secretion is a response mechanism to metal toxicity or purely due to the effects of bacteria. As part of this PhD, a robust sterile method for studying various aspects of seed germination has been developed.

(ii) Careful analysis of changes in the nutrient status of growth media should be carried out focussing on different forms of phosphorus. In addition, synthesis of phytic acid requires a supply of inositol from glucose and the source of this to the germination process needs to be established.

(4) **Investigating the ability of PGPB and legumes in mediating deleterious ethylene levels in plants exposed to metal contamination**

Ethylene is an essential phytohormone that must be secreted at an optimum level for proper plant functioning. The possibility of metal toxicity inducing high ethylene concentration to a level deleterious to plants has been suggested by some authors (Penrose and Glick, 2001, Rajkumar and Freitas, 2008). Some works have suggested that PGPB, through the secretion of the enzyme 1-aminocyclopropane-1-carboxylic acid (ACC*) deaminase,* an enzymes that feeds on ACC the precursor of ethylene, are capable of significantly preventing excessive secretion of ethylene in plants while others have overlooked this as a possible but passive role of PGPB (Belimov et al., 2005, Arshad et al., 2007, Glick et al., 2007). Moreover, some authors have reported possible moderation of ethylene production under metal contamination by cysteine (Goyal et al., 1989, Rawat et al., 2005) while others have suggested ethylene production to be regulating cysteine secretion in plant (Cervantes et al., 1994, Jones et al., 2005).

Measuring ACC deaminase secreting ability of PGPB in nutrient broth has been the major approach to studying this phenomenon and direct measurement of ethylene levels in inoculated plants exposed to metal toxicity is yet to be done (Belimov et al., 2001, Penrose and Glick, 2003). Moreover, it is also likely that mediation of ethylene toxicity in phytoremediating plants through the use of tolerant leguminous plants that naturally harbour diverse species of PGPB in a legume-phytoremediator mixed planting system may be better than the use of PGPB. Direct measurement of the level of ethylene gas emission and correlation with the level of cysteine secretion in plants under bacterial inoculation and legume mixed planting in metal contaminated

environment, may therefore further add to our knowledge of roles of bacteria and legumes in microbial and legume assisted phytoremediation.

### (5) Engineering legume nodulation and bacteria symbiosis in the phytoremediating plant *Brassica juncea*

Despite the results demonstrating their potential effectiveness, the reported remediation techniques are still far from being satisfactory in terms of large scale applications. Firstly, a proper technology for the mass inoculation of *B. juncea* seeds with the identified PGPB will need to be developed. Most importantly, the majority of leguminous plants are edible food/feed crops and using them in toxic metal clean-up may put pressure on their use as food crops. The development of a bio-engineering technique that will endow *B. juncea* plant with legume nodulation and multiple bacterial symbiosis ability, under metal contaminated conditions will therefore be very beneficial. This can be achieved by using state-of-the art biochemical and phytotomics techniques that will potentially involve the following steps:

- Biochemical boosting of flavonoids secretion in *Brassica juncea* plant root: Root secretion of flavonoids triggers secretion of nodulation factors in surrounding bacteria species (Hassan and Mathesius, 2012). Most legumes exude higher amounts of flavonoids than non-leguminous plants. A successful boost of flavonoids in *B. juncea* plant will be a key mechanism in initiating ‘cross talk’ between the plant and PGPB in close proximity.
- Promoting the development of bacterial infection thread in *B. juncea* root: Most PGPB gain access to legumes host cells through tubular cell wall

ingrowths termed infection threads (Van Spronsen et al., 1994). Through these threads, endophytic bacteria (like the *P. brassicacearum* used in this research) can penetrate the plant cell walls and colonise the inner membranes of the plant roots (Brewin, 2004). The genetic sequencing of root cell walls of most leguminous plants has been studied and compared to that of non-leguminous plants. A modification of the *B. juncea* root cell wall sequence can therefore be done to encourage the development of infection thread for the colonisation of growth promoting and metal tolerance bacteria species.

- Introduction of metal-resistance-nitrogenase encoding genes from rhizospheric metallidurans to *B. juncea* plant roots: Plants have been shown to acquire some level of resistance when transformed with genes from metal resistant bacteria strains (Suman et al., 2014). Likewise, transfer of nitrogenase genes from bacteria to plants has been reported to improve plant nitrogen nutrition as well as bacteria diversity (Arthikala et al., 2014). *Rhizobium leguminosarum* (one of the bacteria species used in our experiments) exhibits excellent metal tolerance without losing is plant growth promoting ability (Smith and Giller, 1992). Identification of the gene(s) responsible for both metal tolerance and nitrogenase secretion from the bacteria, and transform the *B. juncea* plant with the isolated gene(s) is also a possible biotransformation mechanism.
- Evaluation of germline transmission of modified character and assessment of toxic metal remediation potential of the first filial (F1) generation plants: The ability of the modified plants to transfer the engineered characters to the seeds will need to be evaluated since most phytoremediating plants are

established by seeds on contaminated soils (Luo and Rimmer, 1995, Dary et al., 2010). The ability of the first filial generation of the transformed plant will also need to be assessed for their ability to hyper-accumulate toxic metals.

- The nature of bacterial colonisation, metal accumulation and speciation in the endowed plants can then be studied with CLSM and synchrotron based XAS and the results compared to the one reported in this research.

The anticipated biochemically endowed *B. juncea* plant will be a complete 'stand-alone' metal remediating plant that will combine the plant growth promoting benefits of PGPB with metal tolerance induced by legumes while maintaining (and most possibly increasing) inherent metal accumulation ability, without the need of bacteria inoculation or a legume.

(6) **From pot experiments to field trials: An assessment of microbial and legume-assisted phytoremediation**

The development of sustainable bioremediation technologies still remains largely a laboratory research or a glass house assessment (Manousaki et al., 2014, Li et al., 2014). Dual microbial inoculation, legume assisted phytoremediation, and legume assisted microbial phytoremediation are all potential sustainable remediation techniques that have been reported through this research to be effective through pot experiments. However, without a long term assessment of these remediation techniques under the influences of real life biological, edaphic and climatic conditions we cannot yet claim a useful remediation technology. It is therefore necessary to assess these remediation techniques in the field. Apart from assessing

the effectiveness of metal remediation in the field, the economics of the proposed techniques will also need to be assessed and be compared to already existing bioremediation technology as well as chemical and mechanical remediation methods.

(7) **Zn bioremediation and biofortification; a complimentary process for remediating Zn contaminated soil and enriching Zn deficient soils**

Although Zn contamination was identified as a major environmental problem in this research, Zn deficiency in arable soils is also a significant global challenge to crop production and human nutrition (Cakmak et al., 1996, Cakmak et al., 1999). PGPB has been reported to enhance Zn bioaccumulation in plants grown in Zn contaminated soil. Apart from bacterial enhanced Zn bioaccumulation, this work also reports PGPB to significantly enhance the accumulation of Zn as stable Zn phytate in plants. The possibilities of harvesting biomass of plants that was inoculated with PGPB to accumulate high amount of Zn as Zn phytate, and using the biomass as organic fertilizer for Zn deficient soil has not been given any research consideration. Using plants used for Zn phytoextraction as a biofortifier of Zn deficient soil may also be a more sustainable alternative (compared to biomass incineration) of disposing plants that have accumulated high amount of Zn.

(8) **Mechanism of plant growth promotion by PGPB under extreme weather conditions - drought and water logging (XAS analysis)**

Some plant growth promoting bacteria have been reported to confer resistance to stress induced by water deprivation in tomatoes and peppers (Mayak et al., 2004b). Amelioration of flooding stress in crops by plant growth promoting bacteria has also been observed (Grichko and Glick, 2001). The explanation of this phenomenon has

however been based purely on the abilities of the bacteria to produce enzymes and hormones that enhanced plant growth under these extreme moisture conditions (Grichko and Glick, 2001, Mayak et al., 2004b). It is however possible that the chemistry of metal and metalloids under these extreme moisture conditions (especially in paddy fields) influences plant growth and it is also likely that the PGPB may be performing other roles relating to changes in the spatial distribution and speciation of micronutrients (e.g. Zn, Cu, Ca, Mn, Mg, Se) within the plant system to promote growth (Ponnamperuma, 1972, Lambers et al., 2008). A synchrotron based XAS analysis of fresh soil, roots and shoots of un-inoculated and inoculated plants in these conditions may therefore give more insight on the mechanism of plant growth promotion by PGPB in water deficient and water logged plant condition. Knowledge acquired will be vital in adapting crop production to climate change.

# Appendix I

## Mechanism behind PGPB induced plant growth promotion and chromium bioaccumulation in *B. juncea* exposed to $Cr^{+6}$

**(Preliminary studies with results)**

### Introduction

Chromium (Cr) is a redox-sensitive transition metal existing in oxidation stages ranging from 0 to +6 (Leita et al., 2011). Cr(III) and Cr(VI) are the most stable oxidation states of chromium in the environment (Kožuh et al., 1999). Cr(VI) is about a hundred fold more mobile and more toxic than Cr(III) (Kim et al., 2002) and exhibits high oxidising potential (Leita et al., 2011). Cr(VI) is a highly poisonous Class "A" carcinogenic metal. The threat from Cr(VI) has been officially recognized by the World Health Organization (WHO, 1988) and it has been formally listed as a human carcinogen (ATSDR 2012). Although deleterious concentrations of chromium contamination from natural sources has been reported in groundwater, e.g. in the Leon Valley in Mexico (Robles-Camacho and Armienta, 2000) and the Sacramento Valley in California USA (Mills et al., 2011), industrial processes such as leather tanning (Bini et al., 2008), stainless steel and alloys manufacturing (Huang et al., 2009), textiles manufacturing (Fibbi et al., 2012), wood preservation (Nielsen et al., 2011), pigments production, and processes in energy generation through the operation of nuclear power plants are responsible for life threatening concentrations of Cr in the environment (Johnson et al., 2006).

Cr(VI) is highly toxic to plants and significantly hinders seeds germination, root/shoot development and may eventually lead to plant death a few days after exposure (Shanker et al., 2005, Rodriguez et al., 2012). Although a few Cr tolerant

plants have been discovered (Ahmad et al., 2013), most plants have been found susceptible to be chromium toxicity at the extremely low concentration of 100 μM $kg^{-1}$ plant dry weight (Davies Jr et al., 2002). Out of 36 plant species evaluated for their chromium remediation potential in soils, *Brassica juncea* (Indian mustard) was ranked as one of the best (Shahandeh and Hossner, 2000). Nevertheless, *B. juncea* is highly susceptible to chromium toxicity at high soil Cr(VI) concentrations.

The use of plant growth promoting bacteria (PGPB) to promote the growth of plants exposed to metal contamination is gaining attention as a method for enhancing metal phytoremediation (Burd et al., 1998, Guo and Chi, 2014). However, little research attention has been given to the use of PGPB to promote the growth and phytoremediation potential of *B. juncea* under chromium (VI) contamination. The role of PGPB in promoting plant growth and enhancing Zn bioaccumulation under chromium (VI) contamination is also unclear.

It was therefore hypothesised that:

(i) PGPB will promote plant growth under Cr (VI) toxicity and increase plant accumulation of chromium.

(ii) Inoculated PGPB will change Cr (VI) speciation in plant roots into less-toxic forms.

**Experimental**

These hypotheses were tested using a factorial, fully replicated experiment in which *Brassica juncea* were inoculated with *Pseudomonas brassicacearum (P)* and *Rhizobium leguminosarum (R)* bacteria and grown on sterile laboratory wiper paper (Tork advanced wiper 420 centerfeed roll M2 System) contaminated with

300 mg $kg^{-1}$of Cr (VI) under aseptic conditions. Whole and cross-sectioned root strands were imaged for bacteria distribution under a confocal microscope and analysed for Cr distribution and speciation on the Beamline I18 two weeks after planting.

**Results and discussion**

A qualitative assessment of plant growth 1 week after planting (Figure AI.1) shows bacteria significantly enhanced the growth of plants exposed to Cr (VI).

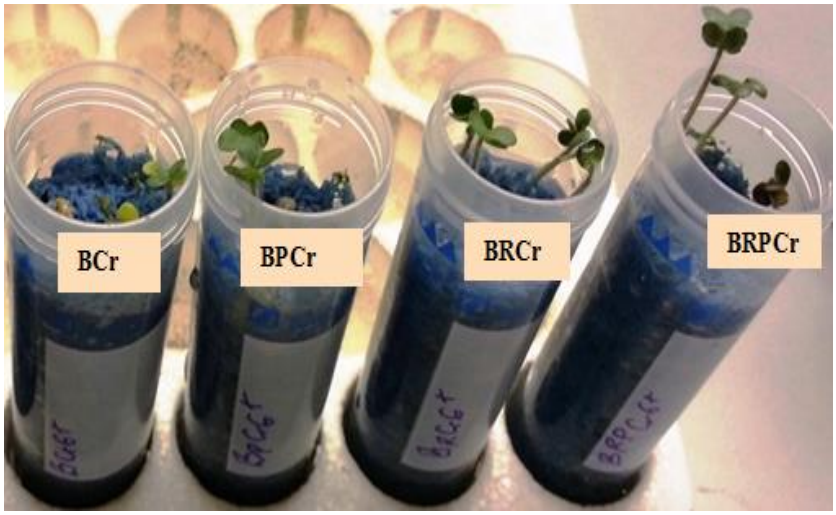



**Figure AI.1: Brassica juncea (B) un-inoculated and inoculated with *Rhizobium (R)*, *Pseudomonas* (*P*) and bacteria combinations (*RP*), exposed to 300 mg/kg of Cr (VI) 7 days after planting**

Un-inoculated plants exhibited stunted growth and yellowish leaf colouration, which are symptoms of Cr (VI) toxicity in plants (Shanker et al., 2005). Meanwhile, plants inoculated with *P* and *R* PGPB significantly ameliorated these toxicity symptoms. A combination of both PGPB (*RP*) produced the best plant growth under Cr toxicity. Moreover, to confirm if the PGPB survived Cr toxicity and are able to colonise plant root, fresh roots were excised 14 days after planting, stained with bacteria Invitrogen LIVE/DEAD Kits and imaged under a confocal microscope. Results of bacteria imaging of the whole plant root (Figure AI.2) shows that un-inoculated plant root (BCr) are free of bacteria cells (the green and red spots in BCR are plant cells not

bacteria cells). Roots inoculated with PGPB however show localised spots of live (see the circled spots in BRCr) and dead (red spots in BRCr) bacteria population. A similar pattern was observed in cross-sectioned root samples.

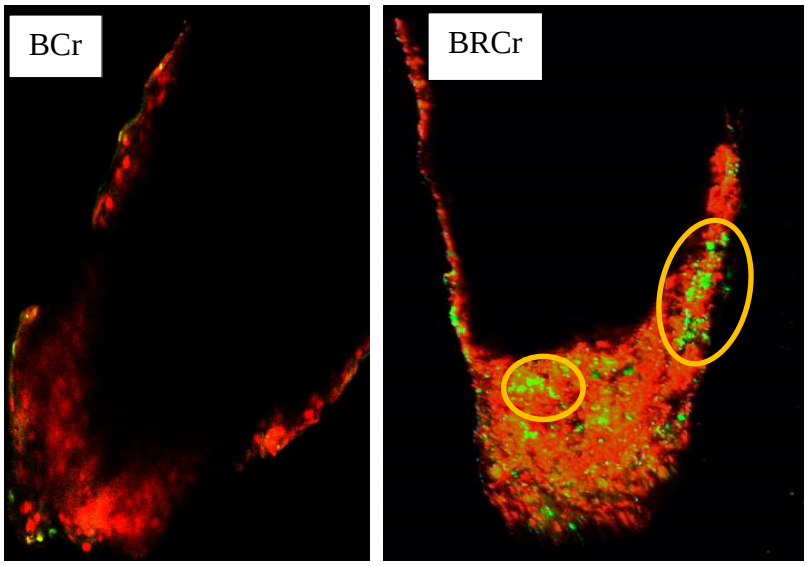



**Figure AI.2: Confocal images of un-inoculated *Brassica juncea* (BCr) & live (green spots) *Rhizobium* bacteria in (BRCr) plant roots.**

Representative μ-XRF maps results (Figure AI.3) clearly show that the presence of inoculated bacteria increased Cr bioaccumulation by more than two orders of magnitude relative to controls, thus significantly enhancing the phyto-extraction of Cr into plant biomass.

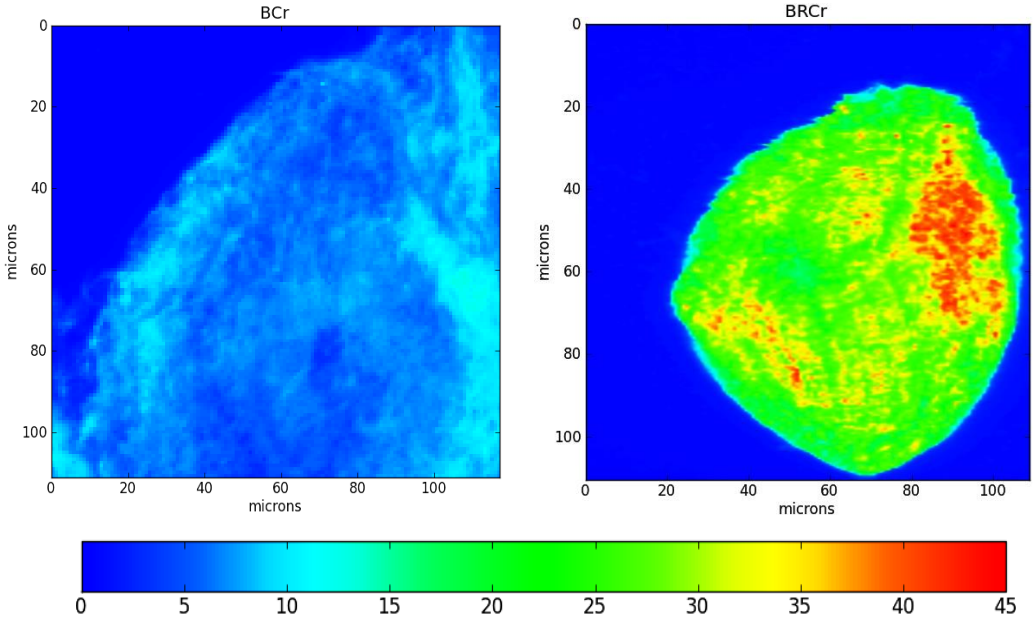



**Figure AI.3: Synchrotron based μ-XRF Maps of Cr distributions in the root of un-inoculated *B. juncea* and plants inoculated with *Rhizobium leguminosarum*. Zn counts are normalized to incoming beam intensity and the beam detector was at the same distance from the sample for the acquisition of the maps. Colour bars (linear scale) indicate Zn counts in plant roots from lowest (blue) to highest (red).**

This result is counter-intuitive since we would expect growth recovery to be associated with Cr being less bioaccessible to the plant.

The paradox of better plant growth in the face of higher Cr uptake was further investigated by probing possible variation in species of Cr in bacteria inoculated and un-inoculated plants using μ-XANES analysis. Cr forms in plants were estimated by comparison to some selected Cr standard compounds (Figure AI.4) using a least-squares algorithm involving Linear Combination Fitting (LCF) in Demeter 0.9.18 2013 version software.

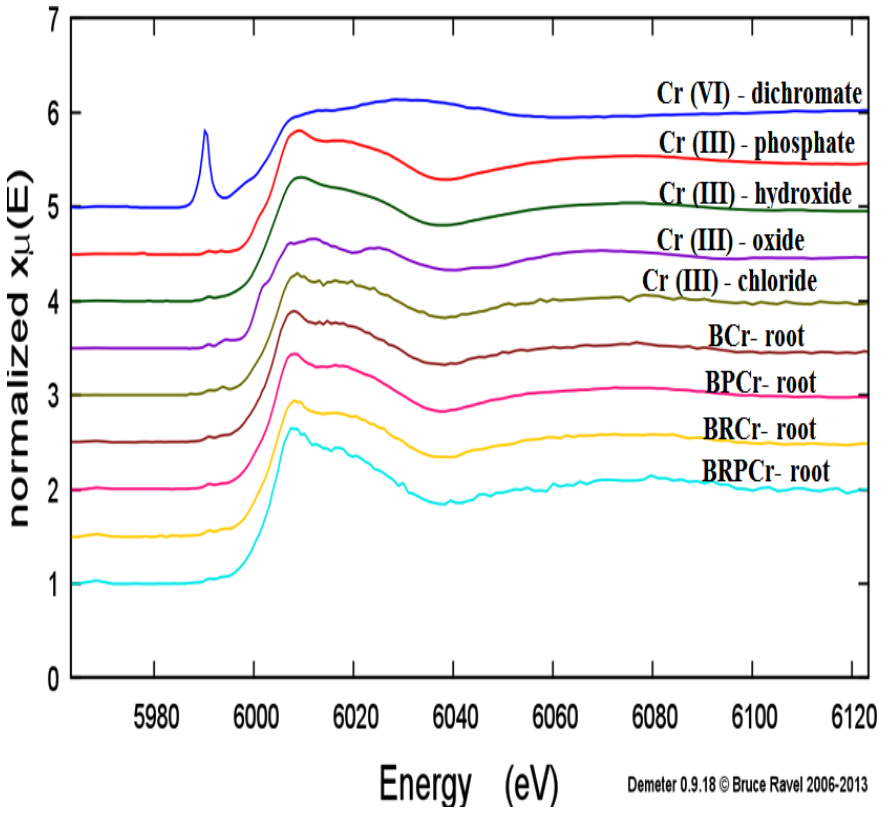



**Figure AI.4: Normalized XANES of Cr in reference standards and root samples**

Unfortunately, the sterile laboratory wiper paper used as growth medium for the experiment appeared to significantly contribute to the transformation of Cr (VI) to Cr (III), based on a quick XAS scan of the paper medium, and subsequently confirmed by leaching tests which showed that ~70% of the added Cr (VI) was in the form of Cr (III). It is therefore not possible to assess the actual Cr (VI) transformation ability of both un-inoculated and inoculated *Brassica juncea* plants, although the fact that there are differences between inoculated and sterile plant growth suggests a role for bacteria in ameliorating toxicity of the remaining 30% Cr that is still Cr (VI).

Moreover, the analysed reference standards (potassium dichromate, Cr chloride, Cr hydroxide, Cr oxide and Cr phoaphate) would appear to be insufficient to fully fit the sample spectra. Nevertheless, Cr hydroxide, Cr phosphate and Cr chloride appear to be major components in the analysed root samples (Figure AI.5).

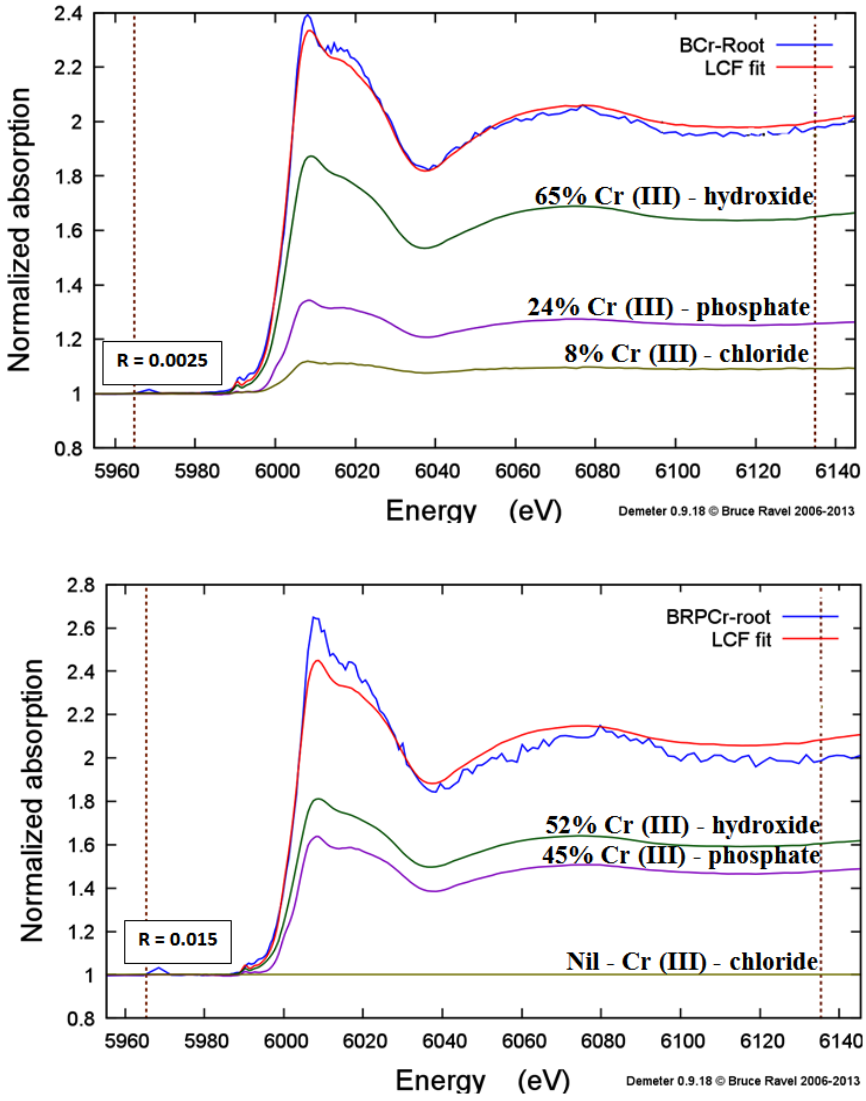



**Figure AI.5: Result of Cr K-edge XANES fitting of un-inoculated *B. juncea* plant root (BZn), *B. juncea* plant root inoculated with both *Pseudomonas and Rhizobium* (BRPZn) and their fractional Zn compound composition. R = $\Sigma_i$ (experimental-fit)$^2$ / $\Sigma_i$ (experimental)$^2$. A lower *R* value means better fit**

The *Brassica juncea* plants did not absorb chromium in the more toxic form of Cr (VI). This may be due to the Cr (VI) transformation effects of the growth media or the inherent ability of the plant to avoid chromium toxicity through the absorption of chromium only in the form of Cr (III). Chromium reduction from Cr (VI) to Cr (III) in plant roots has been reported in *B. juncea* and in *Silene vulgaris* (Bluskov et al., 2005, Pradas del Real et al., 2014).

The better plant growth observed in BRPCr plants may be due to more root accumulation of Cr as Cr (III) phosphate (~45% in BRPCr compared to ~24% in BCr) and the apparent lack of the more soluble Cr (III) chloride, which is present at ~8% in BCr. Cr (III) in very low concentrations is recognized as a plant growth stimulant (Samantaray et al., 1998), and phosphorus is an essential macro nutrient for plant growth (Schachtman et al., 1998). Bacteria induced endogeneous production of Cr (III) phosphate may therefore be a vital biochemical process in plant growth promotion and toxicity ammeliration in plants exposed to Cr (VI) toxicity.

**Conclusion and future work**

Using synchrotron based XAS analysis it was shown that PGPB increase the ability of plants to accumulate metals. Possible bacteria induced changes in Cr speciation in the root of *Brassica juncea* as possible key survival mechanisms in microbial assisted phytoremediation have also been demonstrated. However, it is important to expand the database of reference chromium standards to include chelated forms like Cr phytate, Cr cysteine, Cr histidine, Cr acetate, Cr citrate, Cr oxalate, Cr carbonate and Cr polygalacturonic acid to accurately model Cr forms in the inoculated plants.

In the future, plants grown hydroponically in an aseptic environment will be studied. This will allow complete elimination of possible Cr (VI) conversion to Cr (III). Microtome cryo-sectioned roots samples shall also be analysed in future studies. This will allow clear evaluation of organelle based Cr sequestration within the plant root.

# Appendix II

## Co-localisation of *R. leguminosarum* and Cr (III) in *B. juncea* root

Colonisation of *B. juncea* root by *R. leguminosarum* (green) under Cr (VI) contamination

Localisation of Cr (III) in *B. juncea root inoculated with R. leguminosarum*

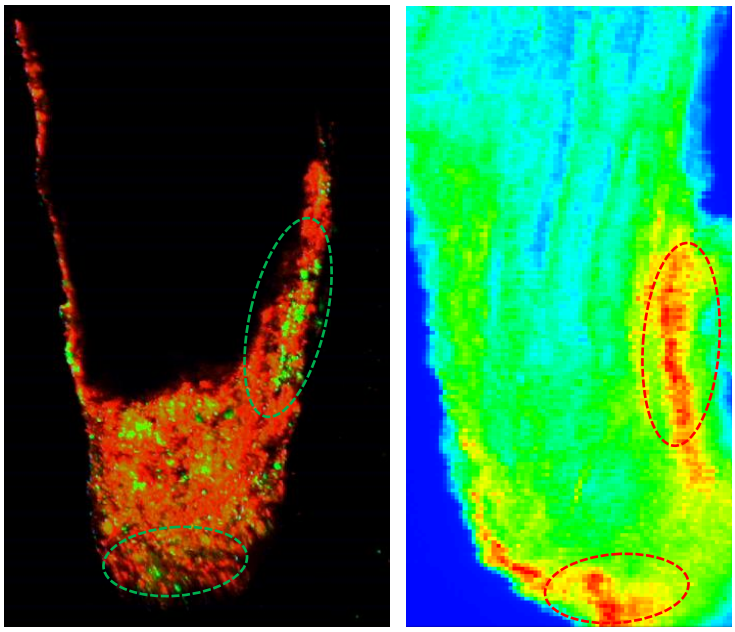


**Figure AII 1: *B. juncea* root inoculated with *R. leguminosarum* and grown in Cr (VI) contamination for 14 days. Same root was imaged by CLSM and synchrotron μ-XRF for bacteria (green circles) and Cr (red circles) co-localisation respectively. Figure shows evidence of bacteria-metal co-localisation**

Animation of 3-D reconstructed images of un-inoculated *B. juncea* root and live (green)/ dead (red) bacteria at the root of *B. juncea* inoculated with *P. brassicacearum* and *R. leguminosarum* under Cr and Zn contamination is available at: https://www.dropbox.com/sh/86n2286kso50t5n/AADWbw37i7dtov0JJaVXLJKNa?dl=0

Or scan bar code for access to images

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